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ANTIMETABOLITES

BY

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Conference Chairman: D. W. WOOLLEY

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BIOCHEMICAL SIGNIFICANCE OF THE COMPETITION BETWEEN *p*-AMINOBENZOIC ACID AND THE SULPHONAMIDES

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Among bacterial growth factors, *p*-aminobenzoic acid is unique in that its biological importance was first recognized, not from any direct effect upon growth, but from its action in overcoming inhibition by sulphanilamide, to which it is chemically rather closely related. This observation led to a hypothesis, since amply confirmed, which explains in biochemical terms the fundamental mode of action of this important group of antibacterial chemotherapeutic agents.[1] Probably as a result, much interest was stimulated in the possible biological effects of analogues of substances important in cell metabolism. This indeed provides the sole justification for the present communication introducing this monograph, for the more general concept, that substances of related structure may compete with others having physiological action, was certainly not new and had been used with profit, for instance, in the field of pharmacology.[2]

The possible value of a substance as a chemotherapeutic agent depends on many other properties besides its *in vitro* antibacterial activity. In particular, it must be selectively toxic to the microbe. It is perhaps not surprising therefore that of the very large number of analogues of other well-established essential metabolites that have now been tried very few show signs of promise in this respect, although very many suppress the growth *in vitro* of microorganisms requiring the parent factor. Nevertheless, a very valuable background of knowledge concerning metabolite/analogue relationships has been built up and has proved, as will be shown in some of the papers presented here, to have a number of most useful and promising applications apart from the chemotherapy of bacterial infections. The Consulting Editor (Dr. Woolley) has always been particularly interested in the fundamental aspects of these matters.

The sulphonamide/*p*-aminobenzoic acid (*p*-AB) relationship has probably been more intensively studied than any other, and this paper will be concerned mainly with biochemical findings and problems arising from such studies.

The Antisulphonamide Activity of p-*Aminobenzoic Acid*

The fact that various cell extracts, autolysates, and enzyme hydrolysates overcome inhibition of bacterial growth by sulphonamides provided an obvious point of attack, from the biochemical standpoint, on the mode of action of these drugs. Woods[1] studied the biochemical and chemical properties of such an antagonist present in yeast and obtained strong presumptive evidence that the active substance was *p*-AB. There was a strict quantitative relationship between the concentration of sulphanilamide added to a culture medium and the amount of *p*-AB (or of yeast

extract concentrate) required to permit growth. One mole neutralized the action of 5,000 25,000 moles of sulphanilamide. These facts, taken in conjunction with the obvious similarity in chemical structure of the two compounds, were strongly reminiscent of the well-known phenomenon of competitive inhibition of enzyme reactions by substances chemically related to the substrate. The following working hypothesis was therefore proposed: (1) *p*-AB or some closely related substance is essential for normal bacterial growth (since an external source is not required by the test organisms used, it is presumably synthesized by them); (2) sulphanilamide, by reason of its chemical similarity to *p*-AB, competes for the enzyme involved in the utilization of the latter by the cell. Such a hypothesis could obviously also cover the closely related case of *p*-AB being a coenzyme or prosthetic group of some essential enzyme system, its union with the enzyme being subject to competition by sulphonamides in the same way.

TABLE 1

MICROORGANISMS FOR WHICH *p*-AMINOBENZOIC ACID IS AN ESSENTIAL GROWTH FACTOR*

Clostridium acetobutylicum	Induced mutants of:
" *butylicum* (one strain)	*Escherichia coli*
" *felsineum*	*Neurospora crassa*
" *thermosaccharolyticum*	*Absidia glauca*
" *kluyveri*	*Ophiostoma multiannulatum*
Lactobacillus (*Streptobacterium*) *plantarum*	
" *arabinosus* 17–5	The growth of a number of other bacteria (*e.g.* several species of *Streptococcus, Cl. botulinum*) is stimulated by *p*-AB.
Leuconostoc mesenteroides Pd-60	
Acetobacter suboxydans 621	
Corynebacterium diphtheriae	
" *gravis* (Dundee)	
Rhodopseudomonas palustris	
Yeasts "45" and "47"†	

* Detailed references are given in a review by Woods.[58]
† Described as single-cell strains isolated from English brewery top fermentation yeasts.

The hypothesis has received its most important confirmation from the fulfillment of the prediction that *p*-AB is an essential metabolite. An external source of this factor has now been shown to be necessary for the growth of a variety of microorganisms, including bacteria, fungi, and yeasts (TABLE 1). Many others which do not require such a source have been found to be able to synthesize it (or material with the same biological activity). The induced mutants provide rather convincing evidence of the status of *p*-AB as a true growth factor, for the same treatment (X rays, mustard gas, *etc.*) also gives rise to mutants requiring one or another of most of the established members of the vitamin B group.

Further confirmation of the hypothesis has been provided by the actual isolation of *p*-AB from yeast[3] and by observations that one factor influencing sulphonamide resistance of some organisms is their quantitative capacity to synthesize *p*-AB or an isotel* (*e.g.*, Landy and Gerstung[4]). From

* Terminology of Williams.[5]

the standpoint of enzyme kinetics, Wood[6] deduced that there should be a constant molar ratio between *p*-AB and sulphonamide if these substances compete for an enzyme. This was shown to be the case with six sulphonamides.

With the sole exception of *Bact. tularense*,[7] the antisulphonamide effect of *p*-AB has been found with all microorganisms tested. Furthermore, the effect of *p*-AB is general with all sulphonamides of the type $NH_2 \cdot C_6H_4 \cdot SO_2 \cdot NHR$ except some sulphanilylanilides.[8] Marfanil and the related "V" drugs are not antagonized by *p*-AB and probably have a different mode of action.[9–11]

Individual sulphonamides may have inhibitory effects on cell reactions not involving *p*-AB. Although these effects may contribute to the overall effect on growth, they cannot explain the main action, since either they are not given by some sulphonamides which are potent inhibitors of growth, or else they are also given by derivatives which have no effect on growth. Thus, sulphanilamide, but not sulphathiazole, inhibits metabolism of CO_2 by Group D streptococci.[12] Similarly, the effect of nicotinic acid in enhancing the respiration of cells of *Shigella paradysenteriae* (Sonne) deficient in this factor is inhibited by sulphapyridine and sulphathiazole but not by sulphanilamide. It is also inhibited by acetylsulphapyridine, which has no effect on growth.[13–15]

The differing relative activities (*in vitro*) of the various sulphonamides against the same species of microorganism are possibly explicable in part by effects such as the above, but probably mainly by physicochemical considerations of structure and ionization in relation to that of *p*-AB (see review by Roblin[16]).

It is not possible here to review all the work on the *p*-AB/sulphonamide relationship which has been carried out since 1940. The work just described certainly justifies the retention of the original hypothesis as a basis for further experiments and interpretations. This will be assumed in the remainder of this review. There remain a few experimental observations that may not appear to fit the simplest form of the hypothesis or may be interpreted in a different way.[17] In considering results obtained with the whole cell, it is always difficult to determine which effects of the sulphonamides are primary. Interference with the metabolism of *p*-AB may well, in turn, affect other cell reactions in which the products of metabolism of *p*-AB are directly or indirectly involved. It is hardly to be expected that all effects of sulphonamides will become clear until the precise function of *p*-AB in cell metabolism is known.

Cell Reactions in Which p-*Aminobenzoic Acid Is Involved*

It may be provisionally accepted that the primary action of sulphonamides is competitive inhibition of an enzyme reaction or reactions in which *p*-AB is concerned either as substrate or coenzyme. The nature of the products of such reactions must next be considered. Evidence has accumulated that *p*-AB functions in some way in the biosynthesis of (a) folic acid, (b) various nucleic acid derivatives, and (c) certain amino acids.

*Folic Acid.** The chemical and biological properties of folic acid and its conjugates and analogues have been the subject of earlier monographs of this Academy[18, 19] and have also been reviewed recently by Jukes and Stokstad.[20] Following the announcement, by Angier and fifteen coworkers,[21] of the synthesis of pteroylglutamic acid (PtG) which had the full biological activity of the liver factor for *Lb. casei* and for the chick, one probable direct function of *p*-AB was at once made clear: the molecule of PtG contains a *p*-AB residue. Earlier work had indeed suggested this possibility. The production in bacterial cultures of material with folic acid activity was found to be influenced by *p*-AB.[22, 23] Furthermore, Miller[24] found the synthesis of folic acid in cultures of *Esch. coli* to be inhibited by sulphonamides. More recently, Sarett[25] has observed that increasing concentrations of *p*-AB increase the production of *Lb. casei* factor by growing cultures of *Lb. arabinosus* (an organism requiring *p*-AB).

Since folic acid is a member of the vitamin B group and an essential metabolite for microorganisms, inhibition of its synthesis might well account for inhibition of growth. Now, if the *only* function of *p*-AB in cell metabolism is for the synthesis of folic acid, and if this is inhibited by sulphonamides, then three things might be expected:

(A) Organisms which require folic acid itself as a growth factor should be resistant to sulphonamides. The metabolic lesion presumed to be induced by these drugs is already present in such organisms.

(B) Folic acid should overcome sulphonamide inhibition of all susceptible organisms. In contradistinction to *p*-AB, this should be in a *noncompetitive* manner, since in this case the product of the inhibited reaction has been provided.

(C) Folic acid should replace *p*-AB for organisms requiring the latter factor.

In considering recent work bearing upon these points it should be kept in mind (for reasons to be stated later) that synthetic pteroylglutamic acid has been used as source of folic acid.

(1) *Organisms requiring or stimulated by folic acid.* Organisms of this type have been examined by Lampen and Jones[26] with regard to points (A) and (B). *Lb. casei*, which requires folic acid, and *Strep. faecalis* Rogers, whose minimal requirement can be met by the slightly simpler compounds, rhizopterin or pteroic acid, are both almost insensitive even to very high concentrations of sulphadiazine. *Strep. faecalis* Ralston and *Strep. zymogenes* 26 C 1 do not require folic acid, but growth is greatly stimulated by it or by *p*-AB. This suggests that the power to synthesize these factors is limiting. These organisms are very sensitive to sulphonamides in the absence of PtG, but in its presence are essentially insensitive, *i.e.*, PtG overcomes inhibition noncompetitively. Furthermore, the concentration of PtG needed was the same as that required by closely related strains for which this factor is essential for growth.

(2) *Organisms requiring* p-*aminobenzoic acid.* The use of such organisms

* "Folic acid" will be used in reference to natural forms of the free factor; "pteroylglutamic acid" will be restricted to the synthetic product.

permits an examination of (C) as well as (B). Two quite distinct types of result have been obtained according to the organism tested.

(a) Pteroylglutamic acid behaves as a noncompetitive sulphonamide antagonist. Nimmo-Smith and Woods[27] find with *Cl. acetobutylicum* (2 strains) and *Sbm.* (*Lb.*) *plantarum* (3 strains) that PtG overcomes sulphonamide inhibition in an essentially noncompetitive manner. The concentration of PtG required over a wide range of drug concentration is not significantly greater than that required for growth in the absence of the drug, *i.e.*, than that required to replace *p*-AB as a growth factor. The molar concentration, however, is 10–100 times greater than that of *p*-AB required for growth. Similar results are reported independently by Lampen and Jones[28] for *Sbm. plantarum* and also for *Lb. arabinosus* (see also Sarett[25]). The relatively low activity of PtG in replacing *p*-AB may be due either to a less efficient absorption of the latter (as suggested by Lampen and Jones) or to a possibility, to be discussed later, that it is not completely identical with the folic acid normally synthesized by these organisms.

A more detailed study of the synthesis of folic acid from *p*-AB by *Sbm. plantarum* has been made by Nimmo-Smith, Lascelles, and Woods.[29] They have been able to obtain synthesis of *Lb. casei* factor by suspensions of this organism in a system containing only buffer, glucose, glutamate, and *p*-AB, and in which no growth occurred. A quantitative relationship was found between the concentration of *p*-AB added and *Lb. casei* factor synthesized. Furthermore, significant synthesis began at just that concentration of *p*-AB which is limiting for optimal growth of this organism. The synthesis was inhibited by sulphonamides but was restored by increasing the *p*-AB concentration, *i.e.*, the inhibition was competitive. Quantitative relationships between the two competitors in this simplified system were closely similar to those obtained by Nimmo-Smith and Woods[27] for the effect on growth.

(b) Pteroylglutamic acid is competitive or inactive as a sulphonamide antagonist. With *Acetobacter suboxydans* 621, Yeast "45" (see TABLE 1), and the induced mutants of *Neurospora crassa* and *Esch. coli*, the concentration of PtG required to replace *p*-AB for growth is proportionately still greater than with the organisms previously mentioned. Furthermore, reversal of sulphonamide inhibition, if it occurs, is competitive.[27, 30, 31] Pteroylglutamic acid appears to act here only as a relatively inefficient source of *p*-AB. It is unlikely that this is due to the known small free arylamine content of most specimens of PtG, since this is mostly *p*-aminobenzoylglutamic acid, which is even less active with these organisms.

(3) *Organisms not requiring* p-*aminobenzoic acid or folic acid.* Lampen and Jones[26] report that they are unable to find noncompetitive reversal of sulphonamide inhibition with *Esch. coli*, *Staph. aureus*, or *Diplococcus pneumoniae*. These organisms, therefore, fall into the same class as the second group of organisms requiring *p*-AB.

(4) *Interpretations.* The work just described is summarized briefly in TABLE 2. With the first group of organisms (those with which PtG shows

noncompetitive sulphonamide antagonism) the results are, in the main, in good agreement with the idea that the chief, if not the only, function of *p*-AB is its requirement for the synthesis of folic acid, and that inhibition of this reaction is the principal point of attack of the sulphonamides on the cell. With the other main group (PtG essentially inactive), no such simple interpretation is available. It is not possible to discern any particular feature of the organisms themselves or the conditions of testing which runs parallel with this differentiation. The basal media for the enterococci and lactobacilli contain amino acids (which, it will be seen later, may be concerned in these matters), but that for *Cl. acetobutylicum* does not, whilst that for *A. suboxydans* (which gives the other type of response to PtG) is again based on amino acids.

TABLE 2

RELATIONSHIP OF PTEROYLGLUTAMIC ACID TO SULPHONAMIDE INHIBITION WITH VARIOUS MICROORGANISMS*

Effect	*Type of nutrition*	*Organism*
Noncompetitive at growth factor level.	Require folic acid	*Lb. casei* *Strep. faecalis* Rogers.
	Stimulated by folic acid and *p*-AB	*Strep. zymogenes* 26 C 1 *Strep. faecalis* Ralston.
	Require *p*-AB	*Cl. acetobutylicum* *Sbm.(Lb.) plantarum* *Lb. arabinosus.*
Competitive or inactive	Require *p*-AB	*A. suboxydans* 621 *Esch. coli* mutants *Neurospora crassa* mutants Yeast "45"
	Do not require *p*-AB or folic acid	*Esch. coli* *Staph. aureus* *Diplococcus pneumoniae*

* For references see text.

Within the bounds of the hypothesis at present under discussion there are several possible explanations for these results.

(*a*) Some organisms may not be able to assimilate preformed PtG. No data is available on this point.

(*b*) PtG may differ slightly in chemical structure from the folic acid required (and normally synthesized) by particular organisms. Some organisms may be unable to convert the former to the latter. There is indeed evidence (summarized and extended by Hall[32]) that folic acid from different natural sources may not be identical. In this connection, it may be noted also that natural *Strep. faecalis* R factor has been identified as a formylpteroic acid (rhizopterin) and has greater growth factor activity than pteroic acid.[33] Again, the analogous formylpteroylglutamic acid is more active than PtG in overcoming growth inhibition of *Strep. faecalis* R by 7-methylpteroylglutamic acid.[34] Finally, the folic acid synthesized by *Strep. faecalis* R from rhizopterin is less stable than PtG.[35]

Another possibility under this general heading is that the final substance with functional activity is an even more complex molecule than PtG* and that the latter is not a normal intermediate in its synthesis by some organisms.

(*c*) *p*-Aminobenzoic acid may have functions other than its requirement for the synthesis of folic acid, or, at any rate, folic acid which is identical with PtG. There is considerable evidence, to be discussed in the next section, that this may be the case and that such functions are also inhibited by sulphonamides.

Forrest and Walker[36] have suggested on the basis of chemical work that the biosynthesis of folic acid and derivatives proceeds from *p*-AB *via* the intermediate formation of a *p*-AB-methylreductone compound. Sulphanilamide forms a similar compound with reductone and may thus block the synthesis of folic acid or lead to the formation of inactive analogues.

Amino Acids and Nucleic Acid Derivatives. Apart from *p*-AB, a number of other substances have been found to have antisulphonamide effects of one type or another. In some cases, the substance alone may overcome sulphonamide inhibition under certain conditions such as a limited range of drug concentration. In other cases, the amount of *p*-AB required to overcome inhibition by a given concentration of sulphonamide may be reduced. Many of these substances have also been shown, either alone or in admixture, to replace *p*-AB for the growth of organisms requiring this factor. Such replacement may be total or only partial. Furthermore, where growth is obtained in the absence of *p*-AB it may be quantitatively inferior. The position is very complex, since a given substance may be active with one organism and inactive (or even synergistic with sulphonamide) with another.

The substances known to have activity in replacing *p*-AB either as growth factor or as sulphonamide antagonist or both are (a) nucleic acid derivatives, *e.g.*, thymine, adenine, guanine, xanthine, and (b) a number of amino acids. Of the latter, methionine has been most frequently reported and with almost every organism tested. Other amino acids which have been implicated are lysine, serine, glycine, allothreonine, threonine, valine, leucine, and perhaps others. In evaluating the significance of a positive result with a single substance, one must bear in mind that the basal medium may have contained other members of these two groups of substances. It is not possible to review this work in full. Detailed references are given by Kohn,[37] Henry,[38] Woods,[39] and Schöpfer,[40] and some more recent findings will be discussed here.

These observations have led to the idea[41, 42] that the substances in question are all products, either directly or indirectly, of the utilization of *p*-AB by the cell, and that such production is inhibited by sulphonamides. This idea is supported by the fact that the concentration of such substances required is at least as great (if not greater) than the concentration needed by other bacteria which have an essential requirement for them. There is no evidence that they function by stimulating the synthesis of *p*-AB.

* This possibility is supported by the discovery (reported while this paper was in press) of the folinic acid group of factors by Shive and coworkers. J. Amer. Chem. Soc. **71**: 3852, 1949.

Two other lines of evidence also suggest that sulphonamides interfere with the synthesis of nucleic acid and its derivatives. First, Schöpfer[43] found that cells of a strain of *Saccharomyces* contained less ribonucleic acid when grown in the presence of sulphathiazole. Secondly, Stetten and Fox[44] showed that an amine accumulated in the medium during sulphonamide bacteriostasis of *Esch. coli* and other organisms. This amine has been identified by Shive *et al.*[45] as 5(4)-amino-4(5)-imidazolcarboxamide. They suggest that it is a precursor of adenine (to which it could give rise by the addition of a single carbon unit, ring closure, and amination in position 6) and that *p*-AB, or some compound synthesized from it, is a coenzyme for the conversion.

The composite effects of nucleic acid derivatives and amino acids are best illustrated by a few recent examples.

(*a*) *Organisms requiring* p-*AB*. Lampen, Roepke, and Jones[30] studied an induced mutant of *Esch. coli*; PtG was ineffective. Almost full growth without *p*-AB was obtained on adding thymine, purines, and an amino-acid mixture (methionine and lysine were the most important of these). Under these conditions, growth could be subcultivated and was highly resistant to sulphonamides. This was also true of the parent strain (not requiring *p*-AB). In Oxford, we have obtained similar findings with other organisms. Thus, with yeast "45," growth (not optimal) in the absence of *p*-AB occurred with a mixture of adenine and several amino acids, of which methionine was essential and leucine highly stimulatory. This mixture was not sufficient to render the organism sulphanilamide resistant although slight growth occurred. Addition of adenine and threonine to a basal medium for *A. suboxydans* not containing them (which supported growth in the presence of *p*-AB) permitted growth without this factor.[46] In the case of *Cl. acetobutylicum*, lysine appears to be an important amino acid, since slow and limited growth occurs in its presence without *p*-AB.[47]

(*b*) *Organisms not requiring* p-*AB*. Shive and Roberts[42] found with *Esch. coli* that the amount of *p*-AB required to antagonise a given concentration of sulphonamide is diminished threefold if methionine is present and another threefold on the further addition of xanthine or guanine. Winkler and de Haan,[48] in an important contribution, carried this matter further and obtained a noncompetitive sulphonamide antagonism with this organism and with *S. typhimurium*. The amount of *p*-AB required for a given concentration of sulphonamide was progressively diminished as methionine, xanthine, and serine were added (in that order). On further supplementation with pteroylglutamic acid (replaceable by high concentrations of thymine), no *p*-AB was required. Growth was slower, however, than with *p*-AB but was increased to almost optimal rate by valine. In the complete mixture, and without *p*-AB, growth was insensitive to the drug. It is suggested, by Winkler and de Haan, that the sulphonamides inhibit at least four enzyme systems involving *p*-AB and concerned respectively in the synthesis of methionine, xanthine, serine, and PtG. It is further suggested that these enzyme systems show decreasing sensitivity to the drug in the order stated.

Discussion

In considering the above results, it should be remembered that *p*-AB *alone* is completely effective both in overcoming sulphonamide inhibition and in promoting growth of organisms which require it. This makes it unlikely that there are a number of distinct effects of sulphonamides on different types of reactions and strongly suggests that they act specifically on an enzyme system or systems involving *p*-AB. There seem to be two main possibilities with regard to the activity of nucleic acid derivatives and amino acids. Their presence may permit an alternative method of growth not involving *p*-AB; there is no evidence on this point. Alternatively, *p*-AB may function in their normal method of biosynthesis and consequently, when they are provided performed, no *p*-AB is required.

If the second possibility is provisionally accepted, there remains the problem as to the precise mechanism by which *p*-AB functions in the various synthetic mechanisms. The function in folic acid synthesis is direct and needs no further comment. In the case of thymine and the purines, there is evidence that the effect occurs *via* folic acid.* Thus, thymine plus purines can replace folic acid both as growth factor and antisulphonamide agent.[49, 50, 26, 28] The high concentrations of thymine required suggest that some derivative rather than the pyrimidine itself is concerned. This is supported by a recent observation[51] that inhibition of the growth of *Leuconostoc mesenteroides* by methylpteroylglutamic acid is overcome by PtG and also by thymidine (or some closely related compound), though not by thymine. A role of PtG in the synthesis of both purines and thymine is supported by the finding of Rogers and Shive[52] with *Lb. casei* that the critical ratio methylpteroylglutamic acid/PtG is increased 2-3-fold by purines and a further 10-fold if thymine is also added. In the full mixture, growth was never completely inhibited by the analogue.

Recently, Prusoff, Teply, and King[52a] have shown that growth of *Lb. casei*, in a medium specifically deficient in PtG, produces cells with a lower content of desoxyribonucleic acid, though not ribonucleic acid.

There is so far no evidence that folic acid functions in the synthesis or metabolism of the amino acids, and thus no evidence that the function of *p*-AB in this case also occurs *via* folic acid. It may be, therefore, that this represents a quite different method of utilization of *p*-AB. Bearing in mind, however, that in most experiments synthetic PtG has been used as source of folic acid and that it is not certain that all natural unconjugated folic acids are completely identical with PtG, it may be justifiable to speculate a little concerning a possible alternative which would provide for a primary utilization of *p*-AB which is analogous in all cases. This would be more in accord with the usual high degree of specificity of function of coenzyme and vitamin-like compounds. The hypothetical scheme given in FIGURE 1 could explain most of the present observations concerning *p*-AB and the sulphonamides.

* Woolley and Pringle have recently found (J. Amer. Chem. Soc. **72:** 634. 1950.) that accumulation of the Stetten-Fox amine also occurs during partial inhibition of *Esch. coli* by 4-aminopteroylglutamic acid, and in similar quantity to when sulphadiazine is used.

It is suggested that there may be three folic acids of biological significance, differing only slightly in chemical structure, but, as a result, possessing different specificities with regard to the substrate with whose metabolism they are concerned as coenzyme or prosthetic group. This would be analogous to the case of the phosphopyridine nucleotide coenzymes, where a difference in structure involving one phosphate radicle only is specifically associated with the division of the dehydrogenase enzyme systems into two main groups. In the case of the proposed folic acids also, it may well be that the particular type of chemical change catalyzed is the same (or very similar) in all the different reactions involved. It is not implied that folic acid itself is a coenzyme. It may well first have to be elaborated to a more complex molecule.* It is suggested that the three folic acids (F^{I}, F^{II}, F^{III}) are necessary for the synthesis of thymine derivatives, purines (or

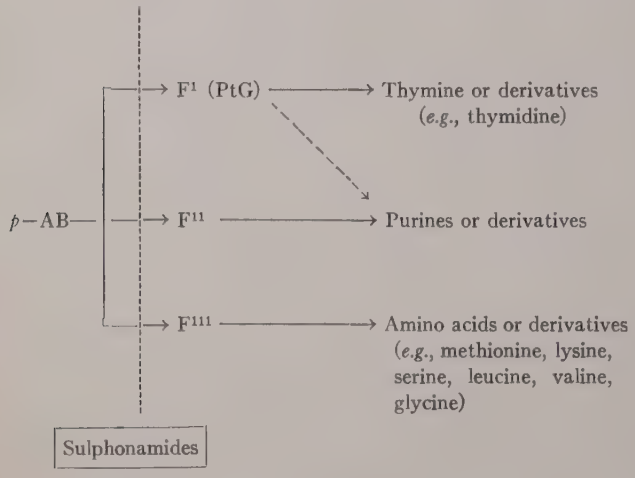

FIGURE 1.

derivatives), and amino acids (or derivatives) respectively. F^{I} is possibly identical with PtG and it is supposed that the synthesis from *p*-AB of all three folic acids is competitively inhibited by sulphonamides. Quantitative differences in the sensitivity of these syntheses to sulphonamide could account for findings such as those of Winkler and de Haan[48] and Shive and Roberts.[42] The different results given by synthetic PtG with various organisms might well reflect a differing ability to convert this folic acid to the other forms or to more complex molecules with coenzyme function. The high intrinsic activity of *p*-AB suggests that in all cases its primary utilization must be of a catalytic nature or lead to products with such activity.

Even on the basis of such a hypothesis, the activity of the amino acids is difficult to understand in certain respects. The particular amino acids

* See footnote p. 1205.

involved (with the probable exception of methionine) vary a good deal from one organism to another. If it is supposed that *p*-AB or a folic acid is concerned in the synthesis of various amino acids, then the most likely interpretation would be that it functions either in some type of chemical transformation common to all these syntheses or in the synthesis of some common intermediate. But it would then be expected that the same amino acids would be implicated with all organisms. An alternative explanation might be that *p*-AB (*via* F^{111}) is concerned not in the synthesis of amino acids but in their further elaboration into peptides. There have been previous suggestions, mostly based on rather indirect evidence, that *p*-AB is concerned in, or sulphonamides interfere with, peptide and protein synthesis.[53–55] Apparent decrease in peptide synthesis in these cases could of course be equally well explained by a decreased synthesis of some of the constituent amino acids. It may be that a folic acid is specifically concerned only in the synthesis of a few amino acids whose limitation would decrease protein synthesis. Other amino acids (in the rather high concentration which seems to be required to give these effects) might then increase the rate of protein and peptide synthesis by a mass action effect.

Effects of Sulphonamides on Respiration. The arguments given by Henry[38] and Sevag[17] that the primary effect of sulphonamides is on bacterial respiration may be reviewed in the light of the recent accumulation of evidence that sulphonamides interfere with the synthesis of nucleic acid derivatives. Since adenine is involved in the structure of several respiratory coenzymes and prosthetic groups, and since some of these (*e.g.*, coenzymes 1 and 11) are known to undergo destruction during functioning which must be made good,[56] it seems more likely that the effect of sulphonamides on respiration is secondary and due to their effect *via* *p*-AB on adenine synthesis.

Selective Toxicity of Sulphonamides. Any final picture of the mode of action of sulphonamides must explain their toxicity to the invading microbe and relative lack of toxicity to the host animal; for upon this difference depends their therapeutic success. The work just outlined throws considerable light on this matter, though there are several outstanding problems. These are discussed in more detail elsewhere.[57]

Folic acid is also a vitamin for animals and birds, but the weight of evidence indicates that they require preformed folic acid and cannot synthesize it from lower compounds such as *p*-AB.[20] They are therefore equivalent to those microorganisms which require intact folic acid and are relatively insensitive to sulphonamides, because the lesion induced by these drugs already exists and the product of the inhibited reaction has to be provided in any event. Since folic acid is an essential metabolite for animal cells, it would be expected that antibacterial substances modeled on folic acid would also be toxic to the animal. It will be seen from later papers in this monograph that this has proved to be the case. Even more interesting is the possible degree of selective toxicity of such analogues as between normal and pathological animal tissues, with which later papers will deal.

Conclusion

Considerable progress has now been made towards an understanding of the function of *p*-AB in cell metabolism and its relation to the fundamental mode of action of the sulphonamides. The main outlines now seem fairly clear, but obviously a great deal of detailed information is still lacking. The problem has become particularly fascinating now that it is clear that it is directly related to mechanisms of biosynthesis, and further developments should throw light on these also.

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THE UTILIZATION OF ANTIMETABOLITES IN THE STUDY OF BIOCHEMICAL PROCESSES IN LIVING ORGANISMS

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The report of Woods[1] in 1940 that the bacteriostatic action of sulfanilamide was competitively prevented by *p*-aminobenzoic acid, which was not known at the time to have a biological function, prompted a widespread search for chemotherapeutic agents among compounds structurally related to metabolites. The theoretical aspects of competitive inhibition of isolated enzyme systems had been previously developed, and in many of these cases, such as malonic acid and related compounds which prevented competitively the action of succinic dehydrogenase on succinic acid,[2] the competitive inhibitors structurally resembled the substrates. The importance, however, of inhibition of enzymatic action by an analogue of the substrate as related to chemotherapy and growth inhibition was not fully realized until this interrelationship of sulfonamides and *p*-aminobenzoic acid was reported.

While much of the early work was directed toward discovery of new and effective chemotherapeutic agents, some investigators have prepared and utilized inhibitory analogues of metabolites in the study of biochemical transformations. Out of these efforts, a new field and new tools for the study of biochemistry have been developed. The term "inhibition analysis" has been used in our laboratory to designate the field of research employing competitive analogue-metabolite inhibitions in the study of biochemical reactions.

The Inhibition Index

The mechanism[3] by which an inhibitor I (analogue) prevents the action of an enzyme E on a substrate S (metabolite) is best illustrated by the following equations, where P represents the product and ES and EI represent the enzyme-substrate complex and enzyme-inhibitor complex, respectively:

$$\begin{gathered} E + S \rightleftharpoons ES \rightarrow E + P \\ E + I \rightleftharpoons EI. \end{gathered}$$

By mass law,

$$\frac{[E][S]}{[ES]} = K_S \tag{1}$$

where K_S is the dissociation constant of the enzyme-substrate complex, and

$$\frac{[E][I]}{[EI]} = K_I \tag{2}$$

where K_I is the dissociation constant of the enzyme-inhibitor complex. By dividing equation 2 by equation 1, one obtains:

$$\frac{[I]}{[S]} = \frac{K_I[EI]}{K_S[ES]}. \tag{3}$$

If $[E_t]$ represents the total enzyme concentration, both free and combined, by definition

$$[E_t] = [E] + [EI] + [ES]. \tag{4}$$

In the application of these equations to biological systems, there are several limitations which greatly simplify the problem. The observable action of the inhibitor is a decreased rate of a biological process, and the specific enzymatic reaction affected by the inhibitor becomes the limiting reaction necessary for obtaining the observable biological effect. In an isolated enzyme system, the observable effect may be a decreased rate of formation of a specific product; the observable effect on bacterial cells or any isolated culture of cells, such as tissue cultures, may be a decreased growth rate or complete inhibition of growth; or the effect observed in an animal or embryo may be in terms of rate of growth, time of survival, or time necessary for the development of certain deficiency symptoms. The simplest method of application of the equations just given to biological systems is the determination of the relationship between the concentration of inhibitor and the concentration of substrate necessary to obtain a defined observable effect within a constant period of time. Other experimental conditions are not allowed to vary.

Under these conditions, the rate, r, of the limiting reaction, can be expressed as follows:

$$r = k[ES], \tag{5}$$

where k is the rate constant for the reaction. Variables which may, under the experimental conditions, affect [ES] are the concentrations of inhibitor and substrate and the total enzyme concentration, $[E_t]$. If the concentrations of inhibitor and substrate are employed at sufficiently high concentrations, so that their utilization by the biological system does not appreciably alter the concentrations, and if the total enzyme concentration, $[E_t]$, is assumed to be constant during the course of the experiment, the variables of equations 3 and 4 are not a function of time. The concentrations of inhibitor and substrate are varied at the outset of the experiment such that the response of the biological system is reduced to a defined amount at the end of a constant period of time. This response is a function of the rate, r, of this limiting enzymatic reaction and the constant period of time used to determine the observable effect of the inhibitor. Since the rate of this reaction is directly proportional to [ES], it follows that [ES] must be a specific concentration, C_{ES}, which will produce the rate giving the defined response in the constant period of time.

If the total enzyme concentration, $[E_t]$, is assumed to be constant in equation 4, [EI] then must be essentially constant, C_{EI}, under the experi-

mental conditions, since [ES] is a defined quantity for a defined growth rate and since [E] approaches 0 for increasing concentrations of substrate and inhibitor, particularly approaching enzyme saturation, and is negligible in comparison with [EI].

Substitution of C_{EI} and C_{ES} for [EI] and [ES], respectively, in the general equation 3 gives for this specific case:

$$\frac{[I]}{[S]} = \frac{K_I C_{EI}}{K_S C_{ES}} = K, \tag{6}$$

where K is the molar ratio of analogue to metabolite in the biological system necessary to obtain a defined inhibition.

The assumption that the total enzyme concentration in the biological system does not vary with changes in the substrate and inhibitor concentrations may not always be valid, even though mother and daughter cells in a growing system would not be expected to vary appreciably in their total enzyme concentration. If the change from the normal presence of optimal quantities of enzyme-substrate (metabolite) complex in the biological system to one of decreased quantities of the complex affects the production of enzyme, the amount of this change would be expected to be a function of [ES] and time. Hence, the total enzyme present at any time could be expressed as follows:

$$[E_t] = [E_t]_0 + f([ES], t), \tag{7}$$

where $[E_t]_0$ represents the total enzyme concentration at the outset of the experiment.

Solution of equation 4 for [EI] and substitution of this value in equation 3 gives the following expression:

$$\frac{[I]}{[S]} = \frac{K_I\{[E_t] - [ES] - [E]\}}{K_S[ES]}. \tag{8}$$

Substituting the value of $[E_t]$ of equation 7 in equation 8, one obtains:

$$\frac{[I]}{[S]} = \frac{K_I\{[E_t]_0 + f([ES], t) - [ES] - [E]\}}{K_S[ES]}. \tag{9}$$

Since the rate of the limiting reaction is proportional to [ES], as indicated in equation 5, r/k could be substituted for [ES] in equation 9. Under such conditions, the only variables included in the equation other than the ratio, [I]/[S], are the rate of the reaction and time, if [E] is neglected as previously indicated. Thus, at any specified time during the experiment, the rate of the reaction is a function of the [I]/[S] ratio. Since the final quantitative response of the biological system is an integration of a function of the rate of this limiting reaction over the constant experimental period, it follows that, in order to reduce the response of the system to a defined value, a specific [I]/[S] ratio is required at the outset of the experiment.

In bacterial growth experiments as well as in other biological systems, this ratio within the cell is a function of the ratio of concentration of analogue to metabolite in the medium. This latter ratio, which is also constant for

a defined inhibition of growth, is called the inhibition index. The inhibition index is therefore related to one single enzyme for which the analogue and metabolite compete, and substances other than the metabolite which exert an effect on the inhibition must act directly or indirectly on this particular enzyme system.

Agents Preventing Analogue Inhibitions

In competitive analogue-metabolite inhibitions of biological systems, exogenous substances, other than the metabolite itself, which are capable of preventing the inhibitory effect of the analogue include: (1) substances increasing the effective concentration of the metabolite; (2) the product or its equivalent of the blocked enzymatic reaction; (3) substances exerting a "sparing action" on this product; (4) agents increasing the effective enzyme concentration; and (5) substances which aid in the destruction of the inhibitory analogue.

(1) *Precursor Effect.* There are several means by which substances may cause an increase in the effective concentration of the metabolite. For example, a limiting precursor may allow an organism to synthesize increased concentrations of the metabolite. Addition of a limiting catalytic factor involved in the biosynthesis of the metabolite may also cause an increase in the concentration of the metabolite which will prevent the toxicity of the analogue. Prevention of destruction of the metabolite by pathways not essential for the response of the test organism may also give such an effect, but it should always be only a moderate effect.

A relatively simple testing technique can be utilized in distinguishing this "precursor" type of effect. This is illustrated in TABLE 1, which indicates the effect of tryptophane on the inhibition of growth of *Escherichia coli* by an analogue of phenylalanine, β-hydroxyphenylalanine.[4] The inhibition index determined at concentrations of phenylalanine above that synthesized by the organism is approximately 1000. Since the organism is inhibited by 300 γ per 10 cc. of the analogue, it appears to synthesize phenylalanine in a concentration equivalent to 0.3 γ per 10 cc. in the medium. Addition of 200 γ per 10 cc. of *dl*-tryptophane allows the organism to grow in concentrations of the inhibitor up to 10,000 γ per 10 cc. If synthesis of phenylalanine by the addition of a limiting precursor were the mode of action of tryptophane, the concentration of phenylalanine being produced under these conditions would be equivalent to 10 γ per 10 cc. in the medium. Hence, at concentrations of phenylalanine above 10 γ per 10 cc., the effect of tryptophane should be negligible, if it is involved in increasing the effective concentration of phenylalanine. As indicated in TABLE 1, the inhibition index, determined with increased concentrations of phenylalanine above 10 γ per 10 cc., is not changed by the addition of the tryptophane.

Thus, the effect of substances increasing the effective concentration of the metabolite can be "diluted out" by increased concentrations of the inhibitory analogue. Increased concentrations of precursors because of mass action tend to have greater effects on the inhibition. In many in-

stances, a precursor of the metabolite may give the appearance of preventing the toxicity of the analogue in a competitive manner.

(2) *Product Effect.* If the product of the inhibited enzyme system can be and is replaced by an external supply, the functioning of the enzyme system is not essential for the biological system. If the analogue does not prevent the combination of the metabolite with another enzyme, it is no longer inhibitory for the biological system as a whole.

TABLE 1

PREVENTION OF β-HYDROXYPHENYLALANINE (PHENYLSERINE) TOXICITY BY PHENYLALANINE AND TRYPTOPHANE*

β-Hydroxyphenylalanine	dl-*Phenylalanine*	*Galvanometer readings*	
		without added tryptophane	*with added* dl-*tryptophane, 200 γ per 10 cc.*
γ per 10 cc.	*γ per 10 cc.*		
0	0	47.0	47.0
100	0	46.0	46.0
300	0	5.0	45.5
1,000	0	2.0	46.0
3,000	0		44.0
10,000	0		2.0
0	3	42.0	48.0
300	3	43.0	47.3
1,000	3	36.0	49.0
3,000	3	5.0	48.5
10,000	3	2.0	2.0
0	10	46.0	47.2
1,000	10	43.5	48.2
3,000	10	30.0	47.0
10,000	10	7.5	4.0
30,000	10	2.0	2.0
0	30	43.0	47.0
3,000	30	41.5	48.0
10,000	30	26.2	32.0
30,000	30	2.0	2.0
100,000	30	2.0	2.0
0	100	46.5	45.9
10,000	100	45.0	46.0
30,000	100	15.5	12.5
100,000	100	2.0	2.0
30,000	300	10.2	
30,000	1,000	16.0	
30,000	3,000	22.0	
30,000	10,000	20.0	
Inhibition index		1,000 Ca.	1,000 Ca.

* Test organism, *Escherichia coli*; incubated 16 hours at 37°.

For example, cysteic acid is a competitive inhibitor of the utilization of aspartic acid in an enzyme system of *E. coli* (inhibition index, 30 Ca.). β-Alanine or pantothenic acid (in amounts normally required by some organisms) prevents the toxicity of any concentration of cysteic acid that aspartic acid can prevent.[5] The results indicate that cysteic acid prevents the conversion of aspartic acid to β-alanine, which is essential for the formation of pantothenic acid or derivatives. Thus, an external supply of the

product or its equivalent may completely prevent the toxicity of an inhibitory analogue for a biological system.

If the metabolite, S, is utilized in the biological system by several enzymes to synthesize several products, P_1, P_2, P_3, *etc.*, a specific analogue, I, may be capable of preventing the conversion of S to one or more of these products. If one of these conversions, for example, $S \rightarrow P_1$, is inhibited to the largest extent and becomes the limiting reaction of the system, equations 1–6 apply to that particular enzyme, E_1, and the K value (becoming K_1 for this particular case) is the inhibition index related to this single enzyme effecting the conversion of $S \rightarrow P_1$.

An exogenous supply of P_1 would completely prevent the toxicity of the analogue if none of the other enzymes, E_2, E_3, *etc.*, were affected. However,

TABLE 2
EFFECT OF PANTOTHENIC ACID ON HYDROXYASPARTIC ACID TOXICITY*

Hydroxyaspartic acid	l(+)-*Aspartic acid*	*Galvanometer reading*	
		without pantothenic acid	*with pantothenic† acid, 5 γ per 10 cc.*
γ per 10 cc.	*γ per 10 cc.*		
0	0	57.5	58.5
10	0	30.0	54.5
30	0	4.0	28.0
50	0		10.0
100	0		4.5
0	30	50.0	52.0
30	30	13.0	53.0
100	30	6.0	51.0
300	30	4.0	13.5
500	30		9.0
1000	30		4.0
0	100	53.5	52.0
100	100	19.0	53.0
300	100	4.0	39.0
1000	100		15.0
2000	100		6.0
Inhibition index		3 Ca.	20 Ca.

* Test organism, *Escherichia coli*; incubated 18 hours at 38–39°.
† β-Alanine (10γ per 10 cc.) replaces pantothenic acid.

the analogue may combine with another enzyme, E_3, for example, and prevent the action of E_3 on the metabolite. The addition of P_1 in excess of the requirements of the biological system does not completely prevent the toxicity of the analogue. On the contrary, this enzyme system, E_3, becomes the limiting process of the biological system and equations 1–6 apply, with K_3, a larger value than K_1, becoming the inhibition index of the biological system in the presence of P_1. In the presence of P_1 and P_3, the analogue either becomes ineffective as an inhibitor of the biological system or, at a still higher inhibition index, prevents another function of the metabolite. P_3 would not be expected to exert any effect on the inhibition in the absence of P_1.

This type of effect is illustrated in TABLE 2, which indicates that hydroxy-

aspartic acid competitively prevents the utilization of aspartic acid in an enzyme system of *E. coli*.[3] The inhibition index is approximately 3. In the presence of pantothenic acid (or β-alanine), the inhibition index is 20. It appears that hydroxyaspartic acid prevents the formation of β-alanine, but it is also capable of preventing another function of aspartic acid at a higher inhibition index.

The effect of addition of the product to the biological system is observed regardless of metabolite concentration, and it either completely prevents the toxicity of the analogue or necessitates a change in the ratio of analogue and metabolite sufficient to equal the inhibition index of another enzyme system utilizing the metabolite. The formation of a series of products of a metabolite may be prevented by an analogue, and, in the absence of one with a lower inhibition index, all the others may not exert any effect on the inhibited system. There is a definite order in which the products must be added in order to demonstrate the effect of each substance.

The general shape of the graph obtained by plotting the response of the system versus increasing concentrations of inhibitor at a constant concentration of substrate is related to the specific enzyme system. The comparison of the inhibition indices for half-maximum and maximum inhibition is often useful in determining whether the effect of a substance which changes the inhibition index is that of a product or of one the related effects subsequently to be described. Two separate enzyme systems affected by the inhibitory analogue of the metabolite would not be expected to have similar dissociation constants, so that any type of data which depends on the dissociation constants of the complexes can be used to show that different enzyme systems are involved.

(3) *"Sparing Effect" on the Product.* If [E] is considered negligible in equation 8, then

$$\frac{[I]}{[S]} = \frac{K_I\{[E_t] - [ES]\}}{K_S[ES]} . \qquad (10)$$

If exogenous substances act in such a manner as to decrease the amount of product P necessary for a defined response of the biological system, then the concentration of the enzyme-substrate complex, [ES], must be decreased by increasing the ratio of inhibitor to substrate, [I]/[S], if the defined response of the system is to be maintained. Since $[E_t]$ is relatively constant and large with respect to [ES] under the testing conditions, it is apparent from equation 10 that the inhibition index would vary approximately inversely with [ES]. Since the amount of the product synthesized is directly proportional to [ES], the decrease in the amount of P required by the organism for the defined response, as a result of the addition of substances exerting a "sparing action," is reflected by practically a proportional increase in the inhibition index.

α-Ketoglutaric acid, citric acid, or *cis*-aconitic acid exerts such an effect on the inhibition of growth of *E. coli* by cysteic acid.[6] The ratio of cysteic acid to aspartic acid necessary for maximum inhibition of growth is 300 in a medium containing these substances but only 30 in their absence. At

very high concentrations of cysteic acid, the amount of pantothenic acid required for prevention of the toxicity of cysteic acid is greatly reduced by the presence of these substances. Hence, their effect is to exert a "sparing action" on the amount of product essential to attain the biological response.

If the product, P, of an inhibited enzyme system is converted to several products, P_a, P_b, P_c, P_d, *etc.*, the influence of these secondary products can be exerted in two ways. If the secondary products are formed by reversible reaction, it is possible that they may even replace P in preventing the toxicity of an inhibitory analogue. In many instances, addition of a limiting secondary product may reduce the amount of P necessary for the defined biological response. In such a case, the "sparing action" on P necessitates an increase in the inhibition index to achieve the same degree of inhibition.

In the application of inhibition analysis to the study of vitamin function, the prevention of coenzyme formation by the analogue of the vitamin may

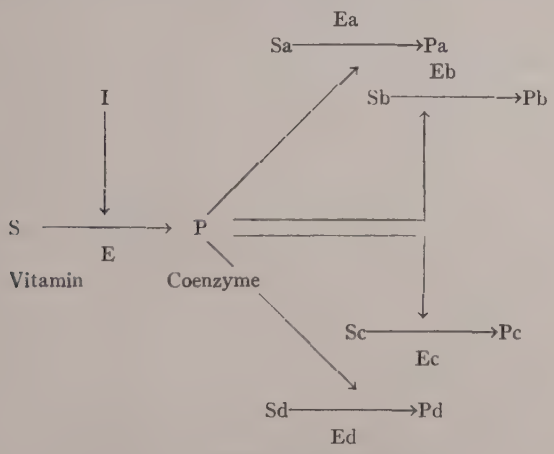

FIGURE 1. Inhibition of coenzyme formation.

occur as illustrated in FIGURE 1. The coenzyme may in turn be utilized in several enzyme systems. Since the apoenzymes corresponding to these enzymes, E_a, E_b, *etc.*, vary in their affinity to combine with the coenzyme and also vary in relative concentration, one particular enzyme system utilizing the coenzyme will become the limiting reaction of the system. The addition of the product, P_a, of such an enzyme, E_a, will then allow the same response of the biological system to be attained on a decreased rate of coenzyme formation which in turn results in an increased inhibition index corresponding to the next enzyme system, E_c for example, which becomes limiting. These secondary products have a definite order in which their effect is exerted. If the organism has a deficient supply of the secondary substrate, S_a, when the formation of P_a is limiting, addition of S_a to the biological system will allow the enzyme to function more efficiently and consequently decrease the requirement for the enzyme, E_a, which in turn will allow a decreased rate of coenzyme synthesis for the same biological

response. This will result in an increased inhibition index. This increase, however, is very moderate in comparison with the increase obtained with the secondary product, P_s.

Other types of "sparing actions" on the product will undoubtedly be found to exist: for example, substances which prevent the utilization of the product in a manner not essential for the biological response.

(4) *Changes in Total Effective Enzyme Concentration.* Substances which cause an increase in the effective total enzyme concentration, $[E_t]$, will affect the inhibition index. Since [ES] is small in comparison with $[E_t]$ and is constant under these conditions, where only $[E_t]$, [I], and [S] are varied, it is apparent from equation 10 that the inhibition index, [I]/[S], will vary approximately directly with $[E_t]$. Hence, substances capable of increasing the effective concentration of the inhibited enzyme will prevent the toxicity of the analogue inhibitor in such a manner as to increase the inhibition index almost proportionally to the increase in the effective enzyme concentration.

Substances which can increase the effective enzyme concentration include a limiting coenzyme or limiting precursors of the coenzyme in cases where the organism contains excess apoenzyme. Also, a limiting intermediate (a limiting second substrate), which is combined by the inhibited enzyme with the substrate (metabolite) to form the product, would also be expected to increase the effective enzyme concentration. This type of effect is usually very moderate in comparison with other types, since such substances are usually furnished in ample amounts by the biological system, particularly growing cells.

(5) *Destruction of the Analogue.* If a substance added to the biological system allows rapid destruction of the inhibitor, it may completely prevent the toxicity of the analogue. If the substance merely increases the rate of destruction of the inhibitor, other effects may be observed.

General Considerations. Since reversing agents of types (2), (3), and (4) are capable of effecting an increase in the inhibition index, a method of distinguishing between these types of reversing agents is desirable. Although it has not been possible to devise a system for isolated cases, a group of agents effecting changes in the inhibition index can usually be differentiated into the various classes.

For example, reversing agents of type (3) and (4) involve changes which affect a single inhibited enzyme, while type (2) involves changes which involve two different enzymes with different dissociation constants. Hence, as previously indicated, data which involve the dissociation constants in their expressed forms will differentiate between substances of type (2) and those of types (3) and (4). Hence, the ratio of the inhibition index for maximum to that for half-maximum inhibition would be expected to change in the presence of an agent of type (2). Reversing agents of type (3) and (4) exert their effects independently of each other, but neither exerts an effect in the presence of the product of type (2).

Another useful testing procedure is the demonstration of synergistic action of two inhibitors, one preventing the conversion of the metabolite to the product and the other preventing a function of the product or some

secondary product derived from the immediate product. The index at half-maximum inhibition may be only a small fraction of that at maximum inhibition. Since the amount of product synthesized during half-maximum inhibition is roughly half that normally synthesized, an inhibitor of the product or some secondary product derived from this immediate product would be expected to reduce the response of the biological system to maximum inhibition at half the index usually necessary. Such a synergistic effect of two inhibitors is useful in demonstrating that certain biochemical reactions are in sequence.

Utilization of Inhibition Analysis in the Study of Intermediary Metabolism

Interrelationships Involving Aspartic Acid. Utilization of inhibition analysis with aspartic acid analogues has resulted in data which indicate the interrelationships shown in FIGURE 2.[3, 5, 6, 7] As previously indicated, both cysteic acid and hydroxyaspartic acid competitively prevent the functioning of aspartic acid in the biosynthesis of β-alanine and pantothenic acid. The β-alanine inhibition index in a salts-glucose medium is 30–100 for cysteic acid and 3–16 for hydroxyaspartic acid. If pantothenic acid or β-alanine is added to the growth medium, cysteic acid does not inhibit growth of *E. coli* at concentrations up to 30 mg. per 10 cc. Hydroxyaspartic acid still prevents growth, however, and the growth inhibition is competitively reversed by aspartic acid, indicating that still another function of aspartic acid is blocked. The inhibition index involving this product is 20–30.

Under the testing conditions with cysteic acid, the rate of pantothenic acid synthesis in *E. coli* is determined by the ratio of cysteic acid to aspartic acid. If pantothenic acid is utilized in the biosynthesis of several secondary products, it would be expected that the various enzymes would differ in their ability to combine the active form of pantothenic acid. The differences in affinity for the coenzyme, as well as the differences in the quantity of each enzyme essential for the response of the organism, would result in one particular secondary enzyme system becoming the limiting reaction on decreasing pantothenic acid synthesis. Addition of the product of this deficient enzyme system would give an effect of type 3 and would result in an increased antibacterial index.

Such an effect is obtained with citric acid, *cis*-aconitic acid, or α-ketoglutaric acid. The inhibition index determined with relatively high concentrations of aspartic acid is increased about 10-fold. Oxalacetic acid and pyruvic acid were ineffective alone, but a mixture of both necessitated a slight increase in the inhibition index. Acetate alone possessed some activity. Pantoic acid was inactive. The sparing action of citric acid on pantothenic acid as demonstrated by the increased inhibition index cannot be accounted for by the precursors of the tricarboxylic acid. Thus, it appears that pantothenic acid-deficient *E. coli* are unable effectively to convert pyruvate and oxalacetate to citric acid or α-ketoglutaric acid.

While this work was in progress, pantothenic acid was identified as a constituent of coenzyme A,[8] which has since been found to function in the oxidation of acetate in yeast.[9]

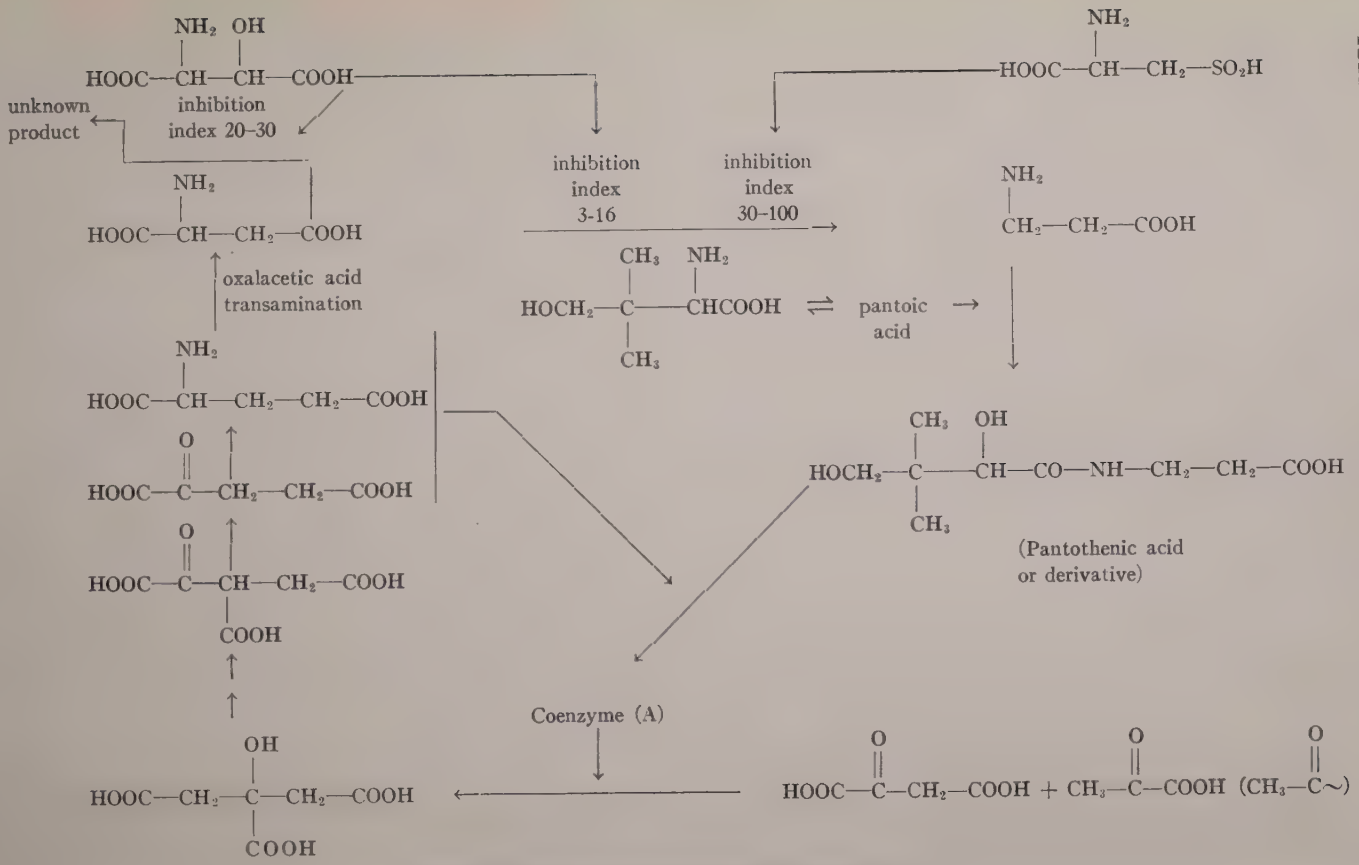

FIGURE 2. Inhibition analysis with aspartic acid analogues (*E. coli*).

The "sparing action" of α-ketoglutaric acid on the system and the precursor effect of glutamic acid in the biosynthesis of aspartic acid by transamination with oxalacetic acid result in a rather unusual effect. Glutamic acid prevents the toxicity of cysteic acid in a competitive manner and is about 3 to 10 times as effective as aspartic acid. This puzzling situation of an apparent precursor of a metabolite being more effective than the metabolite itself led to the elucidation of the complete cycle, in which glutamic simultaneously acts as a limiting precursor and an end product of the blocked enzymatic reaction.

Interrelationships Involving Pantothenic Acid. In *Lactobacillus arabinosus*, *dl*-N-pantoyl-n-butylamine competitively prevents the functioning of pantothenic acid which is required by the organism. Addition of either oleic acid or "Tween 80" to the growth medium resulted in a change in the inhibition index from 3,000 to 30,000. A sample of sodium glycocholate was found to be just as effective as "Tween 80" in exerting this effect. Purification of this sample, however, has resulted in the separation of an active impurity from the sodium glycocholate which was inactive. The active principle appears to be an unsaturated fatty acid. From these data, it appears that pantothenic acid functions in the biosynthesis of oleic acid (or related compounds) from acetate, since the organism requires either acetate or oleic acid for growth.

A strain of *Leuconostoc mesenteroides* requiring pantothenic acid and either acetate or aromatic amino acids for growth is inhibited by *dl*-N-pantoyl-n-butylamine. Pantothenic acid prevented the inhibition competitively. In the presence of acetate, the inhibition index was 300. On the addition of any single aromatic amino acid (phenylalanine, tryptophane, or tyrosine),[10] the inhibition index increased to 3,000. Phloroglucinol, particularly in the presence of increased phosphate, was just as effective as the aromatic amino acids. Sterols such as cholesterol and coprosterone were ineffective in replacing the aromatic compounds but exerted a "sparing effect" on the amount of phloroglucinol necessary to prevent the toxicity of the pantothenic acid inhibitor. It appears from the data that pantothenic acid functions in the conversion of acetate to an intermediate common to the biosyntheses of the aromatic amino acids. Phloroglucinol, which can be considered as a condensation product of three acetate radicals, either is the intermediate or can be converted by the organism to the intermediate.

Certain concentration effects of glutamic acid as compared with *cis*-aconitic acid on the pantothenic acid requirement of *Proteus morganii* suggested the possibility that glutamic acid might be involved in pantothenic acid metabolism in still an additional manner. Accordingly, a number of conjugates of pantothenic acid, including those with all the naturally occurring amino acids and with certain peptides, have been prepared synthetically.[11] Those containing glutamic acid were particularly effective in preventing the toxicity of pantothenic acid antagonists. The results of these and related studies have indicated the involvement of at least one and possibly two other amino acids in the biosynthesis of the pantothenic acid coenzyme (presumably coenzyme A). A naturally occurring conjugate of

pantothenic acid reported to contain glutamic acid has recently been described.[12]

Interrelationships Involving Biotin. The inhibition of growth of *L. arabinosus* by γ-(3,4-ureyleneclohexyl)butyric acid is competitively prevented by biotin. The inhibition index is 30,000. However, addition of either oxalacetic acid or aspartic acid to the medium prevented the toxicity of the inhibitor in such a manner as to increase the inhibition index to 300,000.[13] Sodium bicarbonate and pyruvate also exert an effect on the inhibition index, but their combined action on the inhibition is never as effective as that of oxalacetic or aspartic acid. The addition of both oleic acid and aspartic

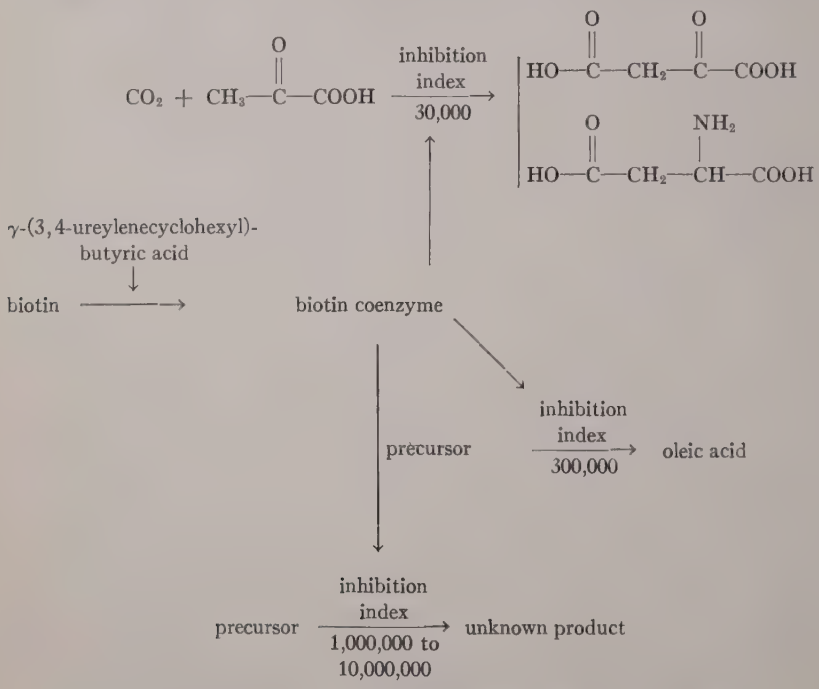

FIGURE 3. Inhibition analysis with γ-(3,4-ureylenecyclohexyl)butyric acid (*L. arabinosus*).

acid to the medium results in an increase in the antibacterial index from 300,000 to a value which varies between 1,000,000 and 10,000,000. Oleic acid alone does not exert any effect. When the inhibition index determined in the presence of aspartic acid and oleic acid is relatively low (1,000,000), *cis*-aconitic acid and related tricarboxylic acids have given variable results in affecting the inhibition. In many instances, these substances are relatively inactive. Thus, it appears that, even if the tricarboxylic acids are essential as products of biotin, still another function of biotin is essential for growth of *L. arabinosus*.

The implications of these data are summarized in FIGURE 3. The analogue prevents the conversion of biotin to a coenzyme functioning in the

carboxylation of pyruvic acid and in the biosynthesis of oleic acid and still an additional unidentified product.

The carboxylation of pyruvic acid as a function of biotin was independently and almost simultaneously discovered in three different laboratories.[14–16] Oleic acid was previously known to replace the biotin requirement of some organisms.[17]

The inhibition of growth of *E. coli* in a salts-glucose medium by 2-oxo-4-imidazolidinecaproic acid is prevented in a competitive manner by desthiobiotin at an inhibition index of 100.[18] At slightly greater concentrations of biotin than the lowest concentration giving any response, the analogue of desthiobiotin does not have any inhibitory effect on growth even at relatively high concentrations. This suggests that the analogue prevents the formation of biotin from a metabolite identical with or similar to desthiobiotin. With the addition of α-ketoglutaric acid or glutamic acid to the medium, the inhibition index is increased to 300. *cis*-Aconitic acid and citric acid did not exert such an effect. By analogy to the carboxylation of pyruvic acid, it would appear that biotin functions in the decarboxylation of oxalsuccinic acid. α-Ketoglutaric acid under such conditions exerts a "sparing effect" on biotin and necessitates an increased inhibition index.

Interrelationships Involving p-*Aminobenzoic Acid and Folic Acid.* The inhibition of growth of *E. coli* by sulfanilamide is related to the inhibition of a series of transformations.[19, 20] As indicated in FIGURE 4, the first limiting transformation is the biosynthesis of methionine presumably from homocysteine, since homocysteine does not affect the inhibition index. The second transformation involves purine synthesis presumably from 5(4)-amino-4(5)-imidazolecarboxamide (or a derivative), since the amine accumulates in the medium under conditions of limiting purine biosynthesis.[21, 22] Addition of glycine to the medium under these conditions results in increased synthesis of the amine.[23] Threonine, though less effective, can replace glycine in obtaining this effect. The inability of serine to replace glycine, particularly in view of a reported mutant of *E. coli*[24] which requires either glycine or serine for growth, suggested that the conversion of serine to glycine was prevented by sulfanilamide.

This is further indicated by the effect of serine in the presence of both purines and methionine on the inhibition index. Folic acid or thymine are somewhat interchangeable in preventing the toxicity of sulfanilamide for *E. coli* when methionine, purines, and serine are added to the medium. The results at this high inhibition index, 100,000, are somewhat variable. For each of the products to exert its effect on the inhibition of growth by sulfanilamide, however, all of the products having a lower inhibition index must be present in the medium.

Adenine usually is not effective in replacing the purine requirement of *E. coli* under these conditions. On the contrary, it is somewhat toxic. Adenosine, xanthine, guanine, and inosine, however, are usually fully effective.

5(4)-Amino-4(5)-imidazolecarboxamide is utilized by *L. arabinosus* and stimulates growth of the organism in a manner similar to purines, but it is required in greater amounts. The amine slowly disappears from the

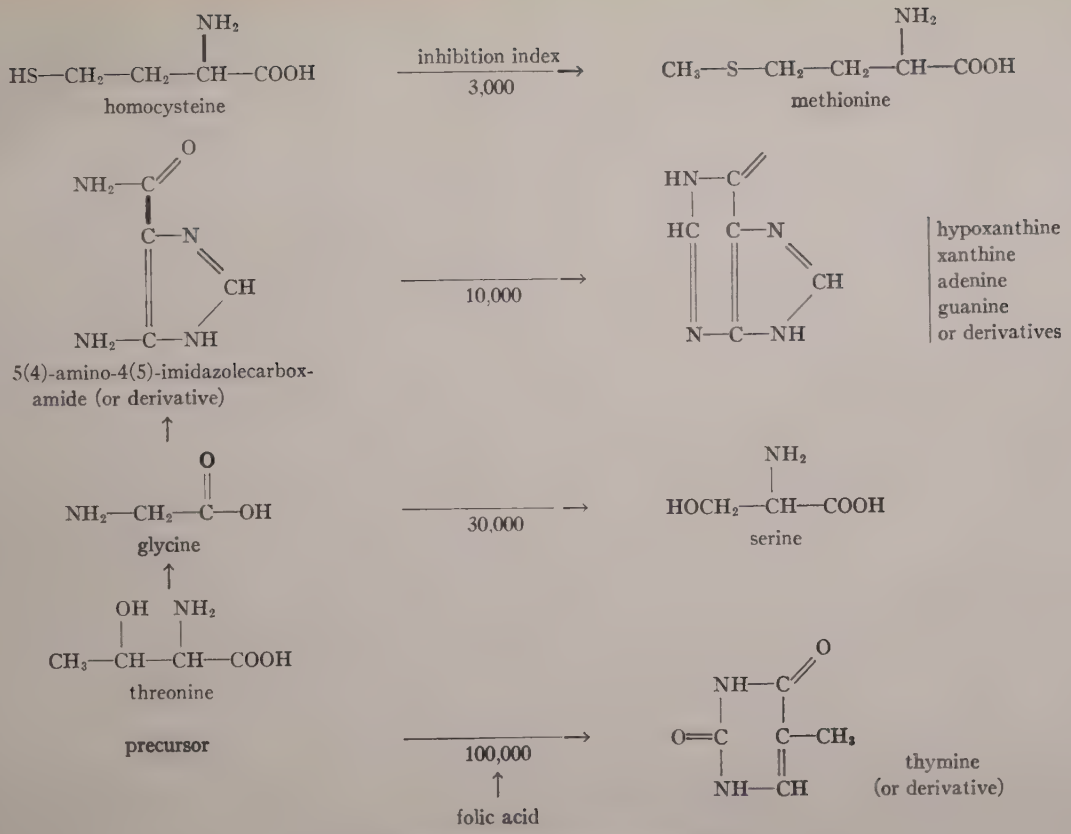

FIGURE 4. Inhibition analysis with sulfanilamide (*E. coli*).

medium under these conditions. Since most organisms cannot utilize the amine, however, it probably is normally conjugated with ribose or desoxyribose during the biosynthesis of the purines or derivatives.

From these results, it appears that *p*-aminobenzoic acid functions in the introduction of single carbon units into purines, pyrimidines, serine (from glycine), and methionine (from homocysteine).

Folic acid is also concerned with the biosynthesis of purines and thymine.[25, 26] In *Lactobacillus casei*,[26] the inhibition index obtained with methylfolic acid as a competitive analogue of folic acid is 30 in the absence of purines, 100 in their presence, and 1000 in the presence of both purines and thymine. Thymine is inactive in the absence of purines.

Since the single carbon unit was involved in *p*-aminobenzoic acid metabolism, a search was made for a folic acid derivative which was capable of serving as a formate carrier. Pteroylhistidine was prepared but did not exert any pronounced activity. The announcement of the structure of rhizopterin,[27] *p*-[N-(2-amino-4-hydroxypyramido-[4,5-b]pyrazin-6-ylmethyl)-formamido]-benzoic acid, gave a clue as to how formate may be carried by a functional derivative of folic acid. Accordingly, formylfolic acid was prepared and found to be approximately 30 times as effective as folic acid in preventing the toxicity of methylfolic acid for *Streptococcus faecalis R*.[28]

Interrelationships Involving Some Amino Acids. The effect of tryptophane on the inhibition of *E. coli* by β-hydroxyphenylalanine has been previously discussed. The same effect was also observed with β-2-thienylalanine. However, it was found that tyrosine prevents the toxicity of β-2-thienylalanine but has no effect on the toxicity of β-hydroxyphenylalanine.[29] The toxicities of both of the inhibitors are competitively prevented by phenylalanine. The inhibition analysis with tyrosine suggested that it was a product of the enzymatic reaction blocked by β-2-thienylalanine.

The toxicity of salicyclic acid for *E. coli* has been found to be prevented by α-amino-β,β-dimethyl-γ-hydroxybutyric acid, which is just as effective as pantoic acid.[7] Since *Acetobacter suboxydans*, which requires pantoic acid or pantothenic acid for growth, could not utilize this amino acid, it seems possible that the amino acid which has been termed "pantonine" may be a normal precursor of pantoic acid.

In a study of the inhibition of *E. coli* by norleucine,[30] methionine prevented the toxicity competitively. Homocysteine and threonine were found to affect the inhibition in a manner characteristic of precursors. Pantothenic acid, thiamin, α-ketoglutaric acid, and glutamic acid were found to be interchangeable in exerting an effect on the inhibition characteristic of substances increasing the effective enzyme concentration of the reaction "blocked" by norleucine. Leucine or a mixture of isoleucine and valine exerted an effect which suggests that methionine functions in the biosynthesis of these amino acids, probably in the amination, since the corresponding keto acids were inactive.

Discussion. While inhibition analysis cannot be used indiscriminately, because of the multiplicity of effects which are obtained, it offers an approach to a systematic study of biochemistry; and, as new testing techniques are

developed, it promises to become the most rapid method of gaining biochemical knowledge.

One recent criticism discussing the limitations of inhibition analysis[31] used the effect of 5-bromouracil on the toxicity of 5-nitrouracil for *L. casei* to illustrate that the data obtained could not be explained by the usual method of analysis. We have confirmed the toxicity of 5-nitrouracil but have found that the toxicity is competitively prevented by uracil rather than folic acid, as indicated in TABLE 3.[32] The inhibition index is approximately 3,000 to 5,000. In the absence of uracil, the ability of folic acid in excess to exert any effect on the system is negligible. In the presence of uracil, however, suboptimal concentrations of folic acid will give responses

TABLE 3
PREVENTION OF TOXICITY OF 5-NITROURACIL BY URACIL*

5-Nitrouracil γ per 10 cc.	*Uracil† γ per 10 cc.*	*Galvanometer reading*	*5-Nitrouracil γ per 10 cc.*	*Uracil† γ per 10 cc.*	*Galvanometer reading*
0	0	67	0	3	89
30	0	59	300	3	69
100	0	30	1000	3	56
300	0	10	3000	3	32
1000	0	5	5000	3	17
0	0.3	76	0	10	88
300	0.3	30	300	10	76
1000	0.3	10	1000	10	70
3000	0.3	7	3000	10	59
5000	0.3	5	5000	10	33
0	1.0	86	0	30	88
300	1.0	60	1000	30	79
1000	1.0	27	3000	30	69
3000	1.0	14	5000	30	53
5000	1.0	8			
			0	0‡	67
			100	0‡	31
			300	0‡	16
Inhibition index, 3000–5000.			1000	0‡	6

* Test organism, *Lactobacillus casei*; incubated 26 hours at 37°.
† Medium contains 0.003γ folic acid per tube.
‡ 1γ folic acid added per tube.

which are similar to those obtained with competitive inhibitions. The involvement of folic acid in the biosynthesis of thymine or an equivalent derivative in *L. casei* has been adequately demonstrated. Hence, it is not surprising that folic acid affects this system, in which thymine is also involved. In the presence of an adqeuate concentration of folic acid, either 5-bromouracil or thymine will completely prevent the toxicity of 5-nitrouracil in a manner which suggests that the "blocked" reaction is no longer essential for growth of the organism. However, 5-bromouracil is not a complete replacement for thymine in preventing the toxicity of methylfolic acid for *L. casei*. These results indicate that 5-nitrouracil prevents the functioning of uracil in a system where thymine can furnish the immediate product of the

system. Uracil and thymine are interchangeable in stimulating the growth of several organisms. Also, 5-bromouracil appears to be able to furnish the immediate product for *L. casei*. That unnatural analogues may function in biological systems in place of the metabolite is well known. Oxybiotin replaces the biotin requirement of several organisms;[33] 2-fluoro-4-aminobenzoic acid can replace the *p*-aminobenzoic acid requirement of *Clostridium acetobutylicum*;[34] one hydroxypantothenic acid has some activity in replacing pantothenic acid;[35] and there are numerous other examples. The demonstration of the activity of an unnatural substance in preventing the toxicity of an inhibitor or in obtaining a growth response of an organism in place of a nutritional factor merely indicates that the equivalent of a natural factor is being supplied. Thus, bromouracil appears to supply the equivalent of the natural product, thymine, under the testing conditions.

The interpretation of inhibition data obtained with substances which act as the equivalent of the immediate products of blocked enzyme systems can be complicated in the study of organisms which are deficient in the biosynthesis of the substrate. For example, if the biosynthesis of the immediate product from a precursor takes place in a conjugated state in a way that the substrate as such is never involved, the addition to the biological system of a precursor or a limiting catalytic factor involved in the biosynthesis of the conjugated form of the substrate may supply the equivalent of the immediate product of the blocked enzyme system utilizing the substrate and completely prevent the toxicity of the inhibitor. For example, it seems possible that the conversion of β-alanine to the pantothenic acid coenzyme may take place without involvement of free pantothenic acid. If such is true, it explains the inhibition data with pantoyltaurine, which prevents the growth of yeast in the presence of pantothenic acid but does not prevent growth in the presence of β-alanine.[36]

Utilization of Inhibition Analysis in the Development of Assays for Naturally Occurring Factors

The Pernicious Anemia Problem. The reports that folic acid was effective in the treatment of pernicious anemia but was not the antipernicious anemia factor(s) of liver extracts have stimulated a large amount of research in attempts to obtain satisfactory assays for the antipernicious anemia principle(s). More than two years ago in the Biochemical Institute, we began a series of studies on factors present in liver extracts used in the treatment of pernicious anemia. Largely through the use of inhibition analysis and related approaches, approximately twenty tests for substances related to *p*-aminobenzoic acid, folic acid, or related factors have been developed.

Thymidine. In the early part of our work, one factor appeared to be very promising, since it prevented the toxicity of methylfolic acid for *Leuconostoc mesenteroides* 8293 as well as the toxicity of either sulfanilamide or 2,4-diamino-6,7-diphenylpyrimido-(4,5b)pyrazine for *L. arabinosus*. The factor was, according to inhibition analysis studies, a product of an enzyme system in which folic acid functioned. It was concentrated approximately 300-fold over autolyzed liver in some experimental liver extracts

used in treatment of pernicious anemia. It was present in an inactive bound form but was rapidly liberated during a very short autolysis at 37°. Concentration of the factor almost 100-fold from a liver extract (30 units per cc.) gave material which effected fair responses with *L. mesenteroides* at 0.05 γ per 10 cc. in a relatively complete medium containing 0.03 γ of folic acid and 200 γ of methylfolic acid per 10 cc.

At this stage, a method of liberation of the factor from liver was developed which increased the yield 10-fold over autolyzed liver. So a concentration procedure was developed directly from pig liver. A colorless crystalline

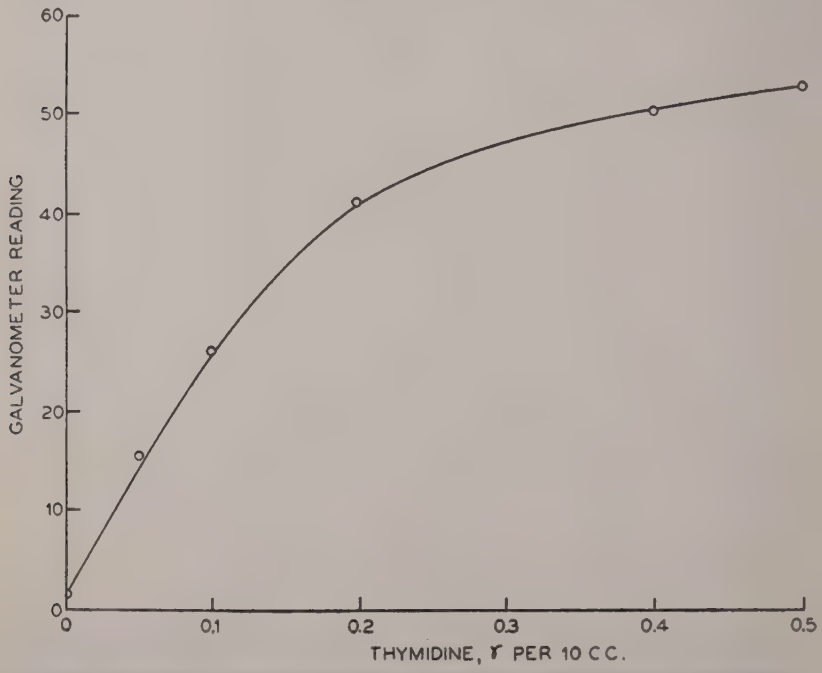

FIGURE 5. Response of *Lactobacillus arabinosus* in 2,4-diamino-6,7-diphenylpyrimido-(4,5b)pyrazine medium.

product was obtained after a concentration of 2,000- to 3,000-fold over liver. This product has been identified as thymidine.[37]

The assay with the pterin inhibitor was used routinely with *L. arabinosus* as soon as it was apparent that the factor giving the response in the test was identical with the factor preventing the toxicity of methylfolic acid for *L. mesenteroides*. The response of *L. arabinosus* to thymidine in the presence of 30 γ per 10 cc. of the pterin inhibitor is shown in FIGURE 5. The shape of the growth curve is more suitable for assay than that obtained with the *L. mesenteroides* test, even though the latter is more sensitive.

Inhibition analysis data indicate that thymidine is a product of the catalytic action of folic acid, since the inhibition index with *L. mesenteroides* increased from 3,000 to 30,000 by the addition of the factor to the medium.

Although thymine and thymidine are interchangeable for some organisms, thymine is inactive under these testing conditions.

Erythrotin. Five of the tests which have been developed were found to respond to identical factors involved in the metabolism of *p*-aminobenzoic acid for *E. coli*.[38] One testing medium contained sulfanilamide in a concentration sufficient to prevent the biosynthesis of methionine in *E. coli*. Using this assay technique, a crystalline, red factor was isolated from liver extracts used in the treatment of pernicious anemia after a 20,000-fold concentration.[39] The response of the organism under our testing conditions to the crystalline factor is shown in FIGURE 6. Because of the distinctive

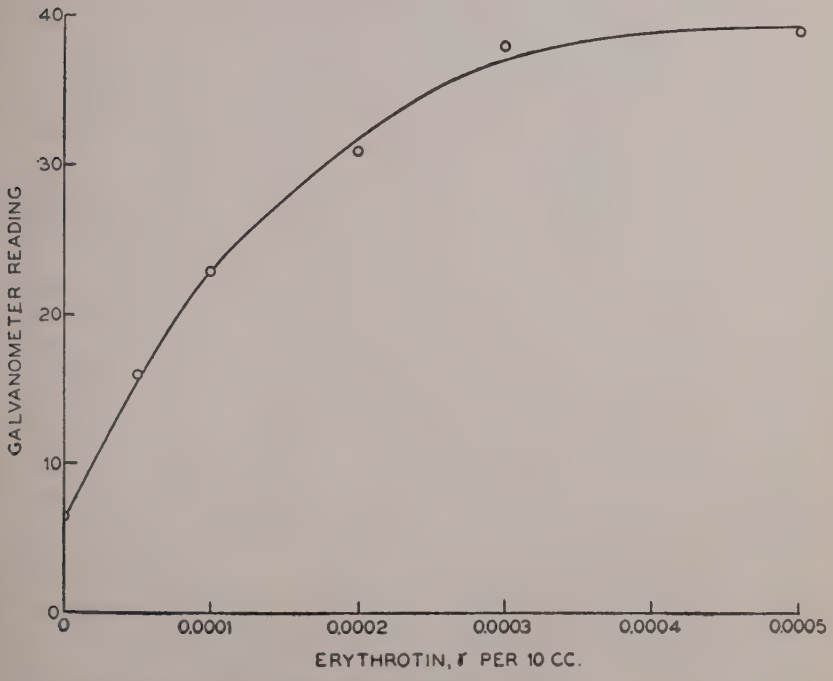

FIGURE 6. Response of *E. coli* in sulfanilamide medium.

color and general properties, the name "erythrotin" seems appropriate for this factor, and we are using this designation until a better name is proposed. Since there is a group of active factors which have closely related biological functions but differ somewhat chemically, it is suggested that these be designated the "Vitamin B_{12} Group," but that individual compounds be given names using the root prefix "erythro-."

Although methionine effectively replaces the factor in the test, erythrotin is about 300,000 times as active; hence, in the isolation or in assay of certain natural extracts, no difficulties have been encountered because of methionine interference.

Whether the biosynthesis of methionine, purines, serine, thymine (or

folic acid), or the unknown additional factor is the limiting reaction resulting from sulfanilamide inhibition of the functioning *p*-aminobenzoic acid, the inhibition index is increased about 3-fold by the addition of erythrotin at a concentration of 0.0005 γ per 10 cc., as indicated in TABLE 4.[40] Increased concentrations of erythrotin even to 0.1 γ per 10 cc. do not enhance this effect. Hence, erythrotin is involved in the biosynthesis of methionine, purines, serine, and folic acid or thymine or their equivalents and at least one additional substance in *E. coli*.

Erythrotin and glutamic acid exert a synergistic effect in preventing the toxicity of sulfanilamide when the biosynthesis of methionine is limiting. Glutamic acid alone is sometimes ineffective, but it usually enhances the effect of erythrotin. Effects similar to that of glutamic acid are obtained with either pantothenic acid or thiamin, both of which are known to func-

TABLE 4
EFFECT OF ERYTHROTIN ON SULFANILAMIDE INHIBITION OF *E. coli*

Supplement	*Inhibition index*	
	without added erythrotin	*with added erythrotin 0.0005 γ per 10 cc.*
None	3,000	10,000
Methionine, 100 γ per 10 cc.	10,000	30,000
Methionine, 100 γ per 10 cc. Xanthine, 100 γ per 10 cc.	30,000	100,000
Methionine, 100 γ per 10 cc. Xanthine, 100 γ per 10 cc. Serine, 100 γ per 10 cc.	50,000–100,000	200,000–300,000
Methionine, 100 γ per 10 cc. Xanthine, 100 γ per 10 cc. Serine, 100 γ per 10 cc. Folic Acid, 0.03 γ per 10 cc.	100,000–200,000	300,000–500,000

tion in the biosynthesis of α-ketoglutaric acid and glutamic acid in *E. coli*.

Still an additional factor, which moves more slowly in various chromatographic separations, is very active in the *E. coli* assay. It is also a red substance, and the name "erythrotide" is tentatively suggested for it. Although it is probable that erythrotin is identical with vitamin B_{12}[41] and the crystalline antipernicious anemia principle isolated by Smith and Parker,[42] it cannot be ascertained with any degree of certainty until these principles are made available for comparisons. It appears probable that "erythrotide" may be the slow-moving red pigment described by Smith.[43] It is interesting to note that a medium including methionine, xanthine, and serine, along with sulfanilamide, allows *E. coli* to respond to either thymine, folic acid, or erythrotin.

Concentrates of erythrotin have been found to reduce the rate of growth of transplant tumors in mice without affecting the body weight of the animals. The animals were first implanted in the right inguinal area with

tumor tissue.[44] After a measurable growth of the tumor had been attained, the concentrate of the factor was given subdermally in the left inguinal area. With a mammary carcinoma, a very crude concentrate containing an equivalent of 5 γ of erythrotin, which was injected every 48 hours for 10 days, retarded growth so that the average size of the tumors was 44 per cent less than that of the controls on the fourth day and 30 per cent at the conclusion of the experiment. Twelve controls and twelve experimental animals were used. With a fast-growing sarcoma, a 1 per cent concentrate containing 5 γ of erythrotin, which was injected daily, reduced the rate of growth of the tumor to approximately 35 per cent that of the controls.

TABLE 5

SPECIFICITY OF VARIOUS ORGANISMS FOR THE VITAMIN B_{12} GROUP AND RELATED FACTORS

Factor	*Organism*		
	*Lactobacillus lactis**	*Lactobacillus leichmanni*†	*Escherichia coli*‡
Erythrotin	+	+	+
Erythrotide	+	not tested	+
Thymidine	+	+	−
Hypoxanthine desoxyriboside	+§	+	−
Ascorbic acid, particularly with aeration	+	+	−
Other O—R poising agents such as —SH, compounds, particularly with aeration	+	+	−
Unknown naturally occurring factor	+	−	−
Methionine	−	−	+

* ATCC 8000.
† ATCC 4797.
‡ Sulfanilamide assay medium.
§ Other purine desoxyriboside also active (private communication, Dr. H. M. Kalchar).

Seven control and seven experimental animals were used. Several other experiments have given similar results. As the agent which affects the growth of tumors is concentrated along with erythrotin, it appears probable that erythrotin is the active principle in the concentrates.

The specificities of various organisms for the "Vitamin B_{12} Group" and related naturally occurring factors are indicated in TABLE 5.[45] It appears that almost any utilizable source of a desoxyriboside can adequately replace the vitamin B_{12} group for both *Lactobacillus lactis* and *Lactobacillus leichmanni*. Also, ascorbic acid, glutathione, and related compounds are able to promote the growth of both organisms. None of these substances are active in replacing erythrotin in the *E. coli* assay.

From these results, it is apparent that inhibition analysis data utilizing natural extracts as source materials can lead to microbiological assays for factors which may be difficult to detect by other means.

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STUDIES ON THE BASIS OF SELECTIVITY OF ACTION OF ANTIMETABOLITES

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It is no longer new to say that slight alterations of the structures of vitamins, hormones, or amino acids frequently lead to the formation of compounds which produce in living things the signs of deficiency of these same metabolites. The studies of the last decade have demonstrated this adequately. These days it is common to synthesize a substance closely related in chemical structure to the next new vitamin, or to some other essential metabolite, and to show that it will inhibit the growth of microorganisms or that it will call forth a specific deficiency disease in animals. The demonstration of the antagonism between the metabolites and their structural analogs can be made not only with living organisms, but also with isolated enzyme systems or with other more or less simplified biological systems. The results of such demonstrations are being used to elucidate the pathways of metabolic reactions as well as to devise new drugs for use in therapeutic experiments and in clinical practice.

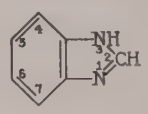
Let us therefore consider for a time the production of a vitamin deficiency state with a structural analog of one of the metabolites, and then proceed to an examination of some of the features of this phenomenon. When mice are given small doses of pyrithiamine, a train of signs of disease is initiated which is very similar to the conditions seen in a deficiency of thiamine.[1] Thus, the incoordination of movement, the convulsions, the retraction of the head over the back, as well as the inanition and failure of growth, which have been considered as the manifestations of this avitaminosis, are seen very clearly in mice treated with pyrithiamine. The effects of pyrithiamine can be prevented or cured by sufficient doses of thiamine, the antagonism between the two compounds being competitive in nature. In other words, the toxic dose of pyrithiamine is not an absolute quantity, but rather is dependent on the amount of thiamine available to the organism. Although 50 micrograms of pyrithiamine will cause fatal thiamine deficiency in a mouse getting 2 micrograms of thiamine per day, 100 micrograms of the analog are needed to achieve the same end when the vitamin intake is 4 micrograms per day. The chemical structures of this pair of antagonistic compounds are shown in FIGURE 1.* The close analogy between them can readily be seen.

The fact that pyrithiamine called forth the easily recognizable sign of thiamine deficiency in animals was of importance. Because of it, the reason for the toxicity of the analog was much easier to appreciate than

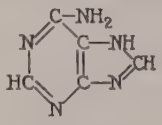
* Some question about the correctness of this structure has been raised by Wilson and Harris in J. Am. Chem. Soc. **71**: 2231, 1949, who found the early preparations of pyrithiamine to be impure. They improved the method of synthesis so as to obtain pure material which they called neopyrithiamine. They expressed the opinion that neopyrithiamine, which has the structure shown in FIGURE 1, was a different substance from pyrithiamine. We have examined this point and have been able to show that the active material of the preparation called pyrithiamine in the older literature is identical with the pure substance renamed neopyrithiamine by Wilson and Harris. In order to avoid confusion, the name pyrithiamine has therefore been retained.

it would have been if the analog were merely a competitive inhibitor of bacterial growth. Pyrithiamine is, in fact, quite active in causing inhibition of growth of those microbial species which require thiamine as a growth factor, but the fact that it produces thiamine deficiency seems more secure when one sees that it elicits in animals the classical signs of the avitaminosis.

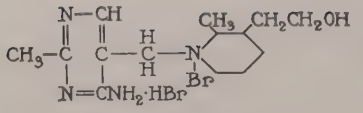
Inhibitory structural analogs, or antimetabolites, have been found for a wide variety of metabolically important compounds. In our own laboratory, such agents have been made which are related to riboflavin, to pantothenic acid, to pyridoxine, to adenine and guanine, to folic acid, to vitamin K, to tocopherol, to ascorbic acid, to thyroxine, and to strepogenin. Other laboratories have swelled this list to large size. These analogs have been



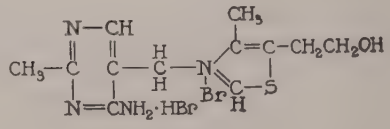
Benzimidazole



Adenine



Pyrithiamine



Thiamine

FIGURE 1. Structure of Pyrithiamine and Thiamine.

found to produce in a variety of living things some or all of the signs of lack of the metabolite to which they bear resemblance.

From the knowledge now at hand, one can see clearly that there are certain general ways in which the structure of a given metabolite can be altered in order to achieve an analog which will be an antimetabolite.[2, 3] These generalizations are illustrated by the examples shown in FIGURE 2. If the metabolite is an acid, the functional carboxyl group may be replaced by certain other more or less acidic groups such as a sulfonic acid radical, a sulfonamide, or even by an aromatic ketone radical. Some very useful pharmacological agents have been made by following this general method (FIGURE 2).

A second general method of arriving at effective antimetabolites can be applied if the metabolite contains a ring system in its structure. Then one or more of the atoms in this ring system may be replaced by some other appropriate atom. This is the type of change involved in passing from thiamine to pyrithiamine, because the sulfur atom of the thiazole ring

of the vitamin has been exchanged for 2 carbon atoms in the analog. In following this general method, carbon atoms may replace nitrogen atoms or oxygen atoms, or these atoms may replace carbons. One atom in the ring system of the metabolite may be replaced by nothing at all, thus leading to the formation of an open chain structure which has antimetabolite proper-

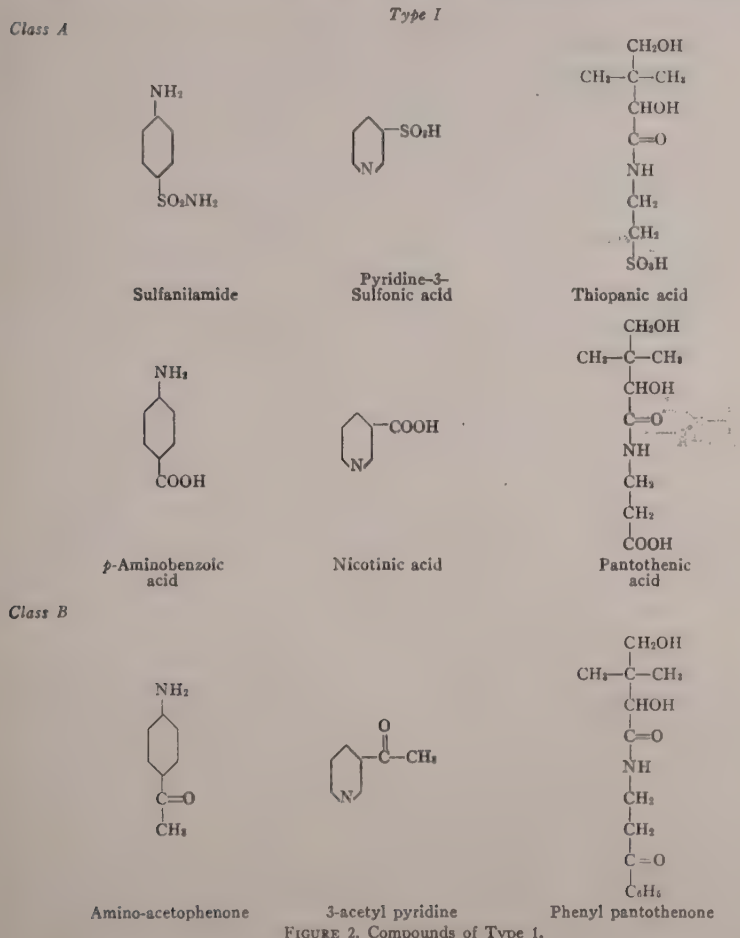


FIGURE 2. Compounds of Type 1.

ties. A few examples to illustrate this general method are shown in FIGURE 3. Because so many metabolically important substances contain ring systems, this has been a widely used method of forming antagonistic analogs.

A third general method is the replacement of alkyl side chains of the metabolite with halogen atoms. Thus, methyl groups may be exchanged for chlorine atoms; and this has yielded some highly active antagonists of

riboflavin and vitamin K (see FIGURE 3). Likewise, hydrogen atoms may be exchanged for fluorine atoms, with somewhat similar results.

These are not the only ways in which the structure of a metabolite may be altered in order to achieve an antimetabolite. Many other methods have been found useful in individual cases. As empirical knowledge increases, more of these general methods will undoubtedly become evident.

One important thing to observe for the purpose of a discussion of selectivity of action of these antimetabolites is that analogs of the same metabolite may be prepared by differing types of structural alteration and that, when this is done, the various kinds of antimetabolite so formed do not always have the same qualitative or quantitative biological effect.

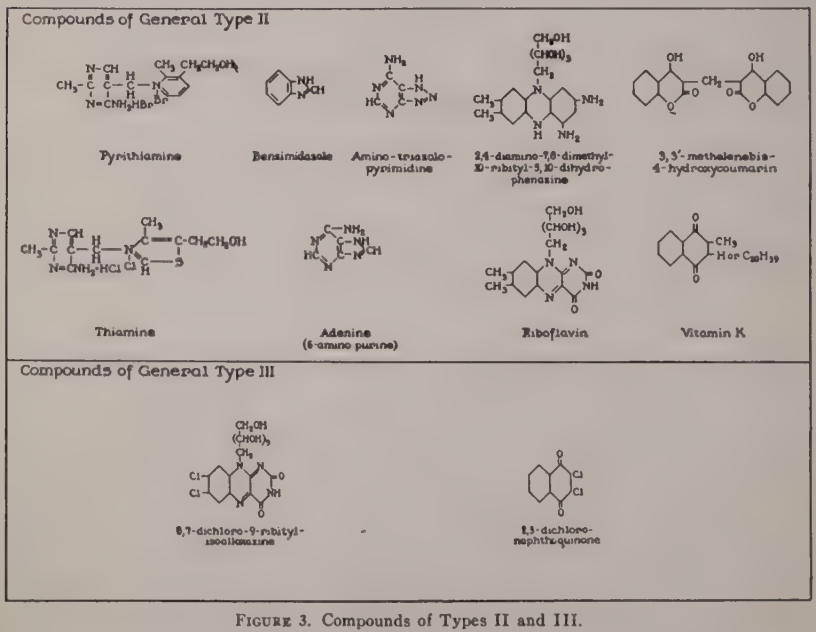
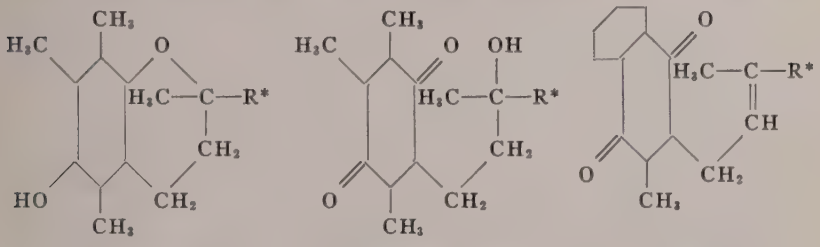


FIGURE 3. Compounds of Types II and III.

Indeed, one kind of analog may affect species of organisms, or tissues of a single individual, which are not susceptible to a second class of analog of the same metabolite. For example, the structure of vitamin K may be changed in at least three ways, with the consequent formation of antagonists to it. First, the alkyl side chains may be exchanged for chlorine atoms. When this is done, a remarkably active inhibitor of fungal growth is obtained.[4] In fact, only 0.002 micrograms of this compound per cc. is needed to inhibit the growth of *Saccharomyces cerevisiae*. The toxic action may be overcome, at least over a limited range of concentration, by small quantities of vitamin K. However, this analog will not cause the signs of vitamin K deficiency in higher animals. An antimetabolite may also be produced by alteration of the ring system of the vitamin. When the carbon atom in position 4 is exchanged for an oxygen atom, and rather marked revision of

the side chains is made, as in dicoumarol (3,3′-methylene-bis (4-hydroxycoumarin)), an analog is obtained which is rather active in calling forth the hypoprothrombinemia characteristic of vitamin K deficiency in higher animals[5, 6] but which is without detectable effect on those fungi which are highly susceptible to the chlorine analog of the vitamin. Similarly, when the ring system of the vitamin is changed by elimination of 2 carbon atoms from the nonoxygenated benzene ring, one arrives at α-tocopherol quinone (see FIGURE 4). This compound will not cause the generalized hemorrhage and the hypoprothrombinemia which result from administration of dicoumarol to animals. Instead, it brings about a hemorrhagic condition which is localized in the reproductive tract of pregnant females. Nonpregnant animals are not affected.[7] This highly selective effect is reversed by administration of small doses of vitamin K.

Thus, we see that a selectivity of action may be realized by discrimination in the manner in which one attempts to form antimetabolites. In studying



* R = $C_{16}H_{33}$

FIGURE 4. Tocopherol quinone structure.

the relationship of structure to activity, I think that this is a fruitful field to investigate further. It may not be surprising that pharmacological agents show selectivity of action, because it is a common finding; but the understanding of the basis of such selectivity seems important, and little known. The discovery of a compound highly toxic *in vitro* to a pathogenic microorganism is of small value if the substance is at the same time poisonous to other living things. Similarly, a drug to combat a disease of noninfectious nature must be able to stop the pathogenic process without unduly affecting normal functions of the organism. Furthermore, the understanding of natural phenomena would be aided considerably if we knew how to inhibit selectively one function of a metabolite without otherwise disturbing the normal metabolic stream. Let us therefore delve into the causes for selectivity of action of some antimetabolites.

The reasons why a substance shows selectivity of action must be quite varied. Some probably have to do with differential solubility or penetrability, but these are not our concern here. Rather, let us examine four situations in which an antimetabolite has shown highly selective effects and in which some slight information has been obtained to show why this should be so.

The first case involves the ability of pyrithiamine to inhibit the growth

of microorganisms. When this antimetabolite is tested with a number of fungi and bacteria, only those species which cannot grow without thiamine, or its component parts, in the medium are found to be susceptible.[8] This fact can be seen readily from the data in TABLE 1. The correlation of nutritional requirement for the vitamin with susceptibility to the analog is striking. Notice that the fungi which need intact thiamine in the medium are inhibited in growth by very small amounts of pyrithiamine, and that those organisms which can grow when merely the pyrimidine moiety of the vitamin is supplied are not so readily affected. The ones which do not require any part of the vitamin for growth can multiply in concentrations of pyrithiamine 500,000 times that which is toxic to the others.

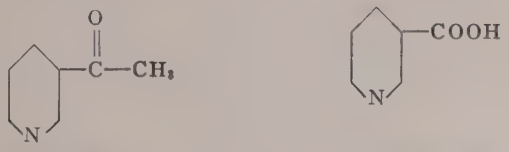
TABLE 1

INHIBITORY POWER OF PYRITHIAMINE FOR VARIOUS MICROBIAL SPECIES

Organism	*Inhibition index pyrithiamine/thiamine*	*Thiamine requirement*
Ceratostomella fimbriata	7	Intact thiamine
Ceratostomella from London plane tree	19	" "
Ceratostomella pennicillata	10	" "
Phytophthora cinnamomi	12	" "
Chaloropsis thielavoides	11	" "
Endomyces vernalis	130	Pyrimidine
Mucor ramannianus	800	Thiazole
Saccharomyces cerevisiae	800	Pyrimidine and thiazole
Staphylococcus aureus	2000	" "
Salmonella gallinarum	1000	" "
Neurospora crassa	Greater than 400,000	None
Escherichia coli	" " 2,000,000	"
Clostridium butylicum	" " 2,000,000	"
Lactobacillus arabinosus	" " 40,000	"
Lactobacillus casei	" " 5,000,000	"
Lactobacillus delbruckii	" " 5,000,000	"
Lactobacillus mesenteroides	" " 5,000,000	"
Lactobacillus pentoaceticus	" " 5,000,000	"
Streptococcus lactis R	" " 5,000,000	"
Propionibacterium pentosaceum	" " 5,000,000	"
Hemolytic streptococcus H69D	" " 4,000,000	"

As is well known, the inhibition index which is used in this table is a measure of toxicity of the analog, because it represents the amount of pyrithiamine needed to inhibit growth half-maximally in the presence of a unit quantity of thiamine. From much previous work, we may conclude safely that those species which do not require thiamine in the medium are capable of making their own supply of it. Therefore, we see that those which can synthesize the vitamin are not susceptible to the antimetabolite, while those which cannot are inhibited in growth by it. The selectivity of action of pyrithiamine is thus correlated with the ability of the organism to synthesize thiamine. Further examination quickly revealed that those species which made their own thiamine possessed an enzyme system which cleaved pyrithiamine at the methylene bridge between the ring systems and

yielded the pyrimidine moiety of thiamine[9] and the pyridine component of pyrithiamine. Those which required thiamine in the medium as a growth factor, and which were affected by pyrithiamine, did not possess this enzyme. Selectivity in this case thus seems to depend on the completeness of the metabolic machinery. The organisms which possess those enzymes associated with thiamine synthesis have one such enzyme with the ability to destroy pyrithiamine. There is reason to believe that thiamine, too, passes through this system and is possibly attacked at the point analogous to that in pyrithiamine.



3-Acetyl pyridine Nicotinic acid

FIGURE 5. Acetyl pyridine structure.

TABLE 2

EFFECT OF 3-ACETYLPYRIDINE AND 3-ACETYLPYRIDINE PLUS NICOTINIC ACID ON GROWTH AND SURVIVAL OF MICE

3-Acetyl-pyridine	*Nicotinic acid*	*Nicotin-amide*	*Animals*	*Deaths*	*Average change in weight*	*Survival time*
mg./day	*% ration*	*% ration*			*gm./wk.*	*days*
0	0	0	10	0	+3.5	>14
10	0	0	19	19		1
4	0	0	24	21		3
2	0	0	4	1		4*
1	0	0	10	0	+1.0	>9
10	2.0	0	3	0	+3.8	>7
10	0.2	0	6	4		1–3*
4	2.0	0	10	0	+3.0	>14
4	0.2	0	10	0†	+2.2	>14
4	0	0.5	9	0	+1.9	>7

* Survival time of the animals that died.
† One of the mice developed redness of the skin on the ventral surface and unkempt hair.

Most cases of selectivity of action in which this effect is correlated with a nutritional requirement for the metabolite probably cannot be explained on this basis. In numerous cases of this sort, the antimetabolite is not destroyed selectively by the species not requiring the metabolite.

The second case which we shall examine is not so readily explained. When the structure of nicotinic acid is changed by replacing the carboxyl group with a methyl ketone, β-acetyl-pyridine is formed. The structures of the two compounds are shown in FIGURE 5. The analog is rather effective in calling forth, in mice or in dogs, the signs of nicotinic acid deficiency.[10] The data in TABLE 2 will illustrate its effect on growth and survival of mice and will also show that nicotinic acid added to the diet will prevent

its toxicity. Although β-acetyl-pyridine is an effective antimetabolite to nicotinic acid when it is tested in animals, it is completely ineffective in a variety of microorganisms. For example, 4 mg. of it per cc. of culture will not affect the growth of *Lactobacillus arabinosus*. This failure is not due to destruction of the agent by the bacteria, because it can be recovered from the culture in which the organisms have grown. There seems little reason to doubt that the analog can penetrate the cells. Thus, to explain its selectivity of action, we must conclude either that the animals have functions for nicotinic acid which the microorganisms lack and with which β-acetyl-pyridine interferes or that the specific proteins with which nicotinic acid reacts in animals differ sufficiently from similarly reactive proteins in bacteria, so that the analog will no longer combine with them.*

The third case of selectivity of action of an antimetabolite deals with an analog which is a large molecule and with a metabolite which, likewise, is a large, nondialyzable, and poorly characterized substance. For this reason, much doubt may exist that we are actually treating of a metabolite and its inhibitory structural analog. Since there is some evidence for believing in this view of the matter, however, and since the findings are of unique character, I feel that we should consider them.

When the influenza virus is mixed with erythrocytes of certain species, such as man or chicken, the virus becomes attached to the cells. If the virus-containing cell-suspension is incubated for a time, however, the virus is set free and appears again in solution. Although the virus is not changed appreciably by this procedure, the cells are changed, because they are no longer capable of reacting with fresh virus. This behavior, along with much supporting evidence, has given rise to the hypothesis that the virus contains an enzyme which reacts with, and destroys, a specific substrate in the susceptible erythrocytes.[11, 12]

A similar substrate is indicated to be present in those cells which the virus invades and in which it subsequently multiplies. At least, the phenomenon of adsorption and elution of the virus may be demonstrated in lung tissue, in which the virus ordinarily grows. The nature of this receptor or substrate is unknown, but the evidence is compatible with the belief that it contains a polysaccharide. If this be true, then a properly constituted structural analog of this substrate should inhibit the enzyme-like attack of the virus upon it. Such an analog should manifest itself by inhibition of the reaction of the virus with erythrocytes and, also, by the inhibition of growth of the virus in susceptible animal cells. The reaction of the virus with erythrocytes is visible, because it results in agglutination of the cells during the time the virus is attached to them. A substance capable of inhibiting the reaction should thus obstruct the clumping of the cells.

A number of polysaccharides of plant origin were tested, and some were found to inhibit the virus-erythrocyte reaction.[13] Apple pectin was one of the most active substances, and data to illustrate its effect are shown in TABLE 3. TABLE 4 reveals which of the polysaccharides tested had

* The underlying mechanism of action of antimetabolites has been discussed in preceding papers in this monograph, so there is no need to elaborate on this point.

activity of this kind. The choice of carbohydrate materials was influenced by the consideration that a polysaccharide, such as the one the influenza virus attacks, which is found in the mucous membranes of the lungs, might very well be composed in part of glucuronic acid residues. Therefore, as analogs, polysaccharides of plant origin which contain uronic acids

TABLE 3

EFFECT OF APPLE PECTIN ON AGGLUTINATION OF CHICKEN RBC IN PRESENCE AND ABSENCE OF INFLUENZA A VIRUS

Apple pectin	*Hemagglutination*	
	With virus	*Without virus*
γ/cc.		
6666	partial	partial
3333	partial	none
1666	partial	none
833	trace	none
208	trace	none
104	partial	none
26	partial	none
13	complete	none

TABLE 4

EFFECT OF VARIOUS CARBOHYDRATE-CONTAINING MATERIALS ON HEMAGGLUTINATION BY INFLUENZA A VIRUS

Substance	*Inhibitory activity*	*Substance*	*Inhibitory activity*
Polysaccharides		*Simple carbohydrates*	
Apple pectin	+	Galacturonic acid	0
Citrus pectin	+	Cellobiuronic acid	0
Flaxseed mucilage	+	Inositol galactoside tartrate	0
Gum acacia	+	Galactose	0
Specific polysaccharide of acacia	0	Aldobionic acid of flax	0
Gum myrrh	+	Glucose	0
Alginic acid	trace	Mannose	0
Soluble starch	0	Ribose	0
"Starch polyaldehyde"	0	*Complex concentrates*	
"Starch polyacid"	0	Blood group A substance	+
Agar	0	Chicken RBC extract	+

other than glucuronic might be effective. The data in TABLE 4 show that galacturonic acid compounds such as the pectins or flaxseed mucilage were capable of inhibiting the virus-erythrocyte reaction. In addition, several other polysaccharides not known to be derived from galacturonic acid likewise were potent. Nevertheless, not all polysaccharides were effective, as witness the result with starch and with the acidic polysaccharide derived from it by oxidation. Alginic acid, a mannuronic acid compound, similarly was not effective.

Since apple pectin and a few other polysaccharides were able to inhibit the reaction of the virus with erythrocytes, they were tested in animal hosts in which the virus multiplies freely. When about ten infective doses of the virus were introduced into the allantoic sacs of embryonated eggs and apple pectin was injected into these sacs either before or after the infection, the multiplication of the virus was largely prevented. The data in TABLE 5 will illustrate this fact. Alginic acid, a polysaccharide which was inactive in the trials with erythrocytes and the virus, likewise proved incapable of inhibiting the multiplication of the virus in the allantoic sacs of embryonated eggs.

Apple pectin was a quite selective agent because, while it prevented the growth of the virus, it did not appear to harm the developing embryos. Large amounts of the pectin could be introduced into the allantoic sac, and, if incubation was continued, apparently normal chicks would hatch

TABLE 5
EFFECT OF APPLE PECTIN AND OF ALGINIC ACID ON MULTIPLICATION OF INFLUENZA A VIRUS IN EMBRYONATED EGGS

Substance	*Amount in mg.*	*When given*	*Eggs*	*Eggs showing virus multiplication*
None			24	24
Apple pectin	50	before virus	61	5
		after virus	56	17
	25	before virus	4	1
Alginic acid	50	before virus	16	14
		after virus	28	24

at the end of the usual period. What is the reason for this selectivity? I believe that it probably resides in the differing function of the responsible metabolite in the host as contrasted to the parasite. Let us therefore examine this metabolite.

In the virus-erythrocyte test system, the working hypothesis which led to the discovery of the inhibitory powers of certain polysaccharides pictured an enzyme in the virus which attacked a specific substrate of polysaccharide nature in the cell. The pectin then acted as an inhibitory structural analog of this metabolite and, by competing with the substrate, was able to prevent the reaction from proceeding. The substrate or metabolite was, however, completely unknown. If the working hypothesis was correct, a test for this metabolite could be devised readily, because, if one merely inhibited the virus-erythrocyte reaction by a minimal quantity of pectin, the introduction of more metabolite into the system should then allow the reaction to proceed. The basic arrangement would be the same as that involved in the cure of pyrithiamine-induced thiamine deficiency of mice by administration of the vitamin, or the counteraction of the inhibitory effects of sulfanilamide on bacterial growth by administration of *p*-aminobenzoic acid. When suitable extracts of the virus substrate were made from erythrocytes, it was indeed found that they would overcome the

action of apple pectin.[14] Because of the high instability of the metabolite, the extraction of it from cells was difficult, but by hemolysis of erythrocytes in the cold an active solution could be made. The effect of this solution in counteracting a small amount of pectin may be seen from the data in TABLE 6. The ability of cells to yield this agent was correlated roughly with their susceptibility to attack by the virus. Furthermore, the antagonism between the metabolite extracted from cells and apple pectin was competitive in nature over the limited range of concentration studied.

With the aid of the test system just described, an attempt was made to purify the metabolite. It was soon found that this could be done if due attention was paid to the unstable nature of the substance. While it remained in the cells, the virus substrate could be stored for several days, but once extracted it disappeared rather rapidly. By alcohol precipitation and by deproteinization with chloroform, a colorless, nondialyzable, highly

TABLE 6

ANTAGONISTIC EFFECT OF A HEMOLYSATE OF CHICKEN ERYTHROCYTES ON THE INHIBITORY ACTION OF APPLE PECTIN TOWARD INFLUENZA VIRUS HEMAGGLUTINATION

Hemolysate		*Apple pectin*	*Phosphate buffer*	*R.B.C. suspension*	*Virus suspension*	*Hemagglutination**
dilution	*cc.*					
		cc.	*cc.*	*cc.*	*cc.*	
0	0	0	0.5	0.25	0	0
0	0	0	0.25	0.25	0.25	c
0	0	0.1	0.15	0.25	0.25	t
0	0.15	0.1	0	0.25	0.25	t
1:10	0.15	0.1	0	0.25	0.25	c
1:1,000	0.15	0.1	0	0.25	0.25	c
1:10,000	0.15	0.1	0	0.25	0.25	p
1:100,000	0.15	0.1	0	0.25	0.25	p
1:10	0.15	0	0.35	0.25	0	0

* c = complete, t = trace, p = partial.

active material was obtained from human erythrocytes. Not only did it antagonize the action of pectin in the hemagglutination reaction, it was also found to react *in vitro* with highly purified influenza virus. This latter reaction was characterized by a loss of the virus substrate and a concomitant decrease in the viscosity of the solution. The material which was isolated, and which had this activity, was probably not a pure compound, but it did contain polysaccharide and gave evidence, as previously cited, that it was a substrate with which the virus reacted.

Consideration of the relationship of this virus substrate to the virus-host interaction leads one to the conclusion that the penetration or invasion of the cell is the most probable site of participation of the metabolite. This opinion was arrived at largely from the more detailed study of virus-host relationship which can be made with bacteriophage and a susceptible bacterium. In the attack of *Escherichia coli* by bacteriophage, one can demonstrate that certain polysaccharides, such as citrus pectin, are able

to protect the host from invasion by the virus.[15] This phenomenon seems to be closely related to the property of apple pectin of protecting animal cells from attack by influenza virus. There are, however, some points of dissimilarity with which we cannot be concerned here. Although space does not permit a detailed recounting of the evidence here, a study of it will reveal that in both the bacteriophage-bacteria system and in the influenza virus-erythrocyte system the polysaccharides act to inhibit invasion of the host cells by the virus. In the case of the influenza virus, it would then follow that this invasion occurs as a result of, or at least coincident with, the destruction of the virus substrate (*i.e.*, the metabolite which has just been described).

This substrate appears to reside at or near the surface of the cells. To the virus, it is a substrate to be cleaved, but to the animal, it is probably a structural unit not engaged in a constant stream of metabolic reactions. If this view of the state of affairs is correct, then the selectivity of action of apple pectin is due to the fact that it competes with a metabolite which the virus must react with in order to continue its multiplication, while in the host this same substance serves a mechanical or structural function and does not act as a substrate for a vital and continuing reaction. A competitor to it therefore does the host no harm.

At the outset of the discussion of this case, some caution was indicated about the inclusion of this example among the instances of metabolites and antimetabolites. This reservation was based on the uncertainty about the chemical structures of the participants. Some investigators prefer to take a quite different view of the mechanism of inhibition of virus action by polysaccharides.[16] Nevertheless, to the present author, the picture of events and participants which has been outlined for the influenza virus seems to concord best with the experimental findings up to the present time. Future evidence may necessitate change in viewpoint.

The fourth case of selectivity of action of an antimetabolite which I should like to discuss is concerned with the sulfonamide drugs. Examination of the facts in this instance will allow us to see the role of differences in metabolic machinery as reflected in nutritional requirements in deciding which organisms will be susceptible and which will be resistant.

Earlier papers in this monograph have shown us how the sulfonamide drugs were found to be structural analogs of the metabolite, *p*-aminobenzoic acid, and how these analogs compete with the metabolite acting as a substrate in an enzyme system. One of the products of this enzyme system seems to be pteroyl glutamic acid. We can see how the metabolite, *p*-aminobenzoic acid, has been built into this new metabolite, pteroyl glutamic acid. By competing with the substrate for this enzyme system, the sulfonamides inhibit the synthesis of pteroyl glutamic acid. These findings and interpretation of them arise from the work of Woods[17, 18] and of Lampen and Jones.[19] These latter authors observed further that those bacteria which require pteroyl glutamic acid as a growth factor are quite resistant to the action of the sulfonamides, whereas many of the species which do not require pteroyl glutamic acid are subject to the action of these drugs. This

is exactly the situation one would expect if the current way of explaining the action of these drugs is correct. Those organisms which synthesize their own pteroyl glutamic acid from *p*-aminobenzoic acid are subject to inhibition by the sulfonamides, and this may be said to be due to a deficiency of pteroyl glutamic acid so produced. On the other hand, those species which have no enzyme system for the synthesis of pteroyl glutamic acid, and hence require it as a growth factor, do not possess the metabolic function which the drugs inhibit and thus are resistant to the action of these agents. Therefore, the ability of a sulfonamide drug to inhibit the growth of an organism would seem to depend on the possession of a metabolic system for the formation of pteroyl glutamic acid, and this in turn is reflected by the lack of a nutritional requirement for that vitamin.

The application of this interpretation to an understanding of the selectivity of action of the sulfonamide drugs seems evident.[20] Many pathogenic bacteria have no nutritional need for pteroyl glutamic acid, while many kinds of animals do require this vitamin. One may then say that the animal host is not readily harmed by the drug because it lacks the system which is affected by the sulfonamide, while the parasite is retarded because it depends on this metabolic system for multiplication. It is fortunate indeed that many kinds of microorganisms can synthesize pteroyl glutamic acid.

Although this explanation of the selectivity of sulfonamides has much to recommend it, there are several points in the argument at which the data are insufficient to be convincing. Let us take note of some of these. (1) While animals such as chickens, guinea pigs, monkeys, and dogs have been shown to require pteroyl glutamic acid when tested in the usual type of experiment to demonstrate nutritional deficiency diseases, other animals such as rats and mice must be subjected to unusual conditions before the need for the vitamin can be shown. Thus, it has not been definitely established that all animals cannot synthesize pteroyl glutamic acid. Some species may have a limited capacity to make this vitamin. All that can be said is that several species of animals do not have this ability sufficiently well developed to meet all their needs. (2) Although a few bacterial species have been found in which the inhibition of growth caused by sulfonamides may be overcome completely and in a noncompetitive fashion by pteroyl glutamic acid, this is not true for most organisms. Perhaps this situation contributes materially to the selectivity of action of sulfonamides or, indeed, to their ability to control infectious diseases at all. If pteroyl glutamic acid in small amounts readily overcame the action of the drugs in those bacteria which do make their own vitamin, enough of it could probably be found in the tissues of the host to nullify the therapeutic effects of the drugs. Nevertheless, this situation is a difficult one to explain completely with the existing hypothesis.

Because some uncertainty exists about the correctness of the hypothesis to explain the selectivity of the sulfonamide drugs, and because the understanding of this problem is important, we have attempted to test this explanation by applying it to another metabolite. For this purpose, one requires knowledge of a vitamin for which the biological precursors are known, just as the precursor of pteroyl glutamic acid is known to be *p*-aminobenzoic

acid. One should then construct a suitable analog of this precursor. This task should not be difficult, because we have seen earlier in this discussion that generalizations are available to guide such an undertaking. If the hypothesis is correct, the new analog should produce signs of deficiency of the vitamin in those living things which do not require it and should not affect those which do have need for it. It was intended to present the complete data in this paper, but, owing to difficulties encountered in the synthesis of the analog, only preliminary results are now available.*

In presenting these four cases of selectivity of action of antimetabolites, I have attempted to state briefly the facts as determined by experiment, and then to attempt an explanation of them in so far as it can be made with existing knowledge. Necessarily, opinions have entered rather deeply into these explanations; therefore, they should be regarded as working hypotheses set up as points of departure for further study. Such future studies seem justified not only as a means of investigation of metabolic reactions, but also because the very soul of chemotherapy, whether of infectious or of noninfectious diseases, is selectivity of action. I think we should have a better understanding of it.

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* Since this paper was read, the experiments have been completed. The analog is ϵ-(2,4-dichloro-sulfanilido)-caproic acid, which is derived from the metabolite pimelic acid by the exchange of one carboxyl group for the dichloro-sulfanilido radical. This substance was shown to inhibit the growth of several species of bacteria which do not require biotin. It did not harm several species which could not synthesize this vitamin and hence possessed a nutritional requirement for it. In the first class of organisms, the action of the analog was antagonized competitively by pimelic acid and noncompetitively by biotin. These demonstrations, therefore, add support to the hypothesis just described.

ION ANTAGONISM IN BACTERIA AS RELATED TO ANTIMETABOLITES

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In 1882, Ringer[1] showed that a solution of sodium chloride would not maintain the beat of a heart perfused with it unless additions of calcium and potassium chlorides were made. This initial observation of what is now called "ion antagonism" was extended to intact animals by Loeb,[2] to plants by Osterhout,[3] and to bacteria by Flexner,[4] Eisler,[5] and Lipmann.[6] Subsequently, results of a large number of investigators (cf. Falk[7]) have emphasized the importance of maintaining the proper ratio of ions for the proper functioning of biological systems, and a host of "antagonistic" or "synergistic" relationships between individual ions in particular biological systems has been observed.

The trend of thought concerning ion antagonism can be summarized in the statement by Falk in 1922[7] that "the effect exerted by electrolytes appears to be primarily an effect upon external or internal membrane surfaces and upon surface interphases in colloidal systems." Few attempts at a more exact delineation of the mechanism of ion antagonism in living organisms have been made. Since knowledge of the inorganic requirements of the biological systems studied was either limited or nonexistent, no attempts to relate ion antagonism to nutritional requirements of the organism were made. Indeed, Loeb[8] discounted the possibility that physiologically balanced ion solutions had nutritional significance, on the grounds that certain fish studied by him survived for long periods of time either in distilled water or in a solution containing correct proportions of NaCl, KCl, and $CaCl_2$, but not in "unbalanced" solutions of these ions.

It is the thesis of the present paper that many cases of ion antagonism can be explained on nutritional grounds, *i.e.*, that an ion which suppresses growth frequently does so by interfering with one or more of the essential metabolic roles played by an ion required for growth. Since the nutritionally essential trace elements function at least in part as necessary components of metabolically essential enzymes, a more exact picture of the mechanism of action of antagonistic ions might be to visualize a competition between the antagonists for an enzyme surface. An enzymatically active metalloprotein (M_EE) results from the normal combination of the nutritionally essential ion (M_E) and the apoenzyme (E); an enzymatically inactive metalloprotein (M_TE) results when the "toxic" ion (M_T) is thus combined. If combination of both metals with the protein is readily reversible, as diagrammed here, then the extent to which the enzyme can function (and growth of the organism proceed where the functional enzyme is required for growth) will depend only upon the ratio of M_E to M_T and not upon the absolute concentration of either, and a true competitive type of inhibition will result. Noncompetitive and intermediate types of inhibition could also result where reaction (2) was irreversible or only partially reversible.

$$M_E + E \rightleftharpoons M_EE \quad \text{(active)} \qquad (1)$$

$$M_T + E \rightleftharpoons M_TE \quad \text{(inactive)} \qquad (2)$$

Our experiments on ion antagonism in the lactic acid bacteria, discussed below, are fully consistent with this explanation and are most readily explained in terms of it.

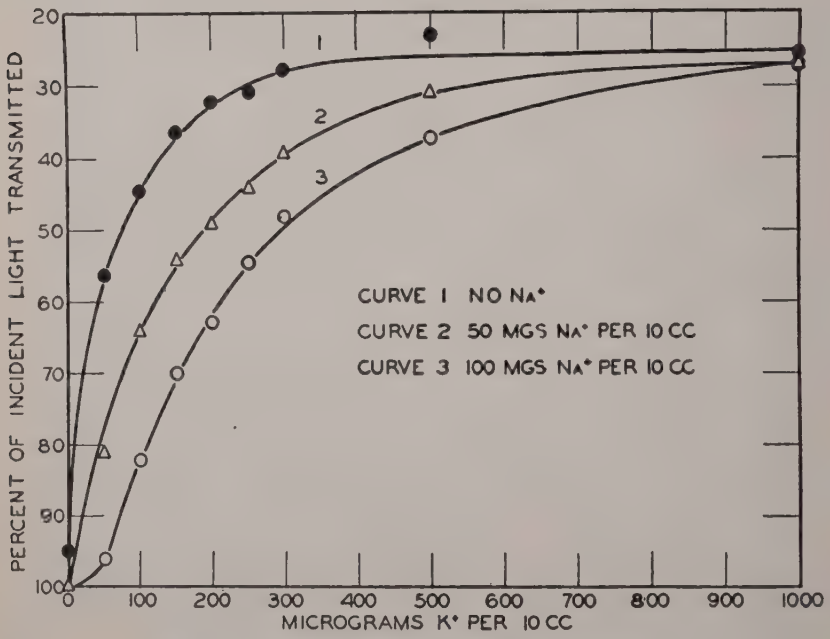

FIGURE 1. The effect of Na^+ on the response of *Lactobacillus casei* to K^+.

The Effect of Ions Related to K^+ (cf. Ref. 9). In a suitable potassium-free medium which is also low in sodium and ammonium ions, little or no growth of any of the lactic acid bacteria occurs unless K^+ is added.[9] With this medium, the effect of additions of Na^+ on the growth response of *Lactobacillus casei* to K^+ was determined, with the results shown in FIGURE 1. In the presence of low concentrations of K^+, Na^+ inhibits growth. This inhibitory action is overcome, however, by an increase in the concentration of K^+. As the amount of Na^+ present is increased, the amount of K^+ required is correspondingly increased.

Four other organisms tested showed similar behavior. The molar ratios

of Na^+ to K^+ at which half-maximum growth occurred are listed for several different concentrations of Na^+ in TABLE 1. At the higher concentrations of Na^+, these ratios approach the constant values indicative of a true competitive relationship between the two ions. In terms of the diagram pre-

TABLE 1

MOLAR RATIOS OF Na^+ TO K^+ PERMITTING HALF-MAXIMUM GROWTH AT VARIOUS LEVELS OF ADDED Na^+

	mg. Na^+ per 10 cc. medium			
	25	*50*	*75*	*100*
	$[Na^+]/[K^+]$ for half-maximum growth			
L. arabinosus	472	620	725	765
L. casei	530	640	877	895
S. faecalis	340	530	688	805
L. mesenteroides 9135	—	1890	2120	1990

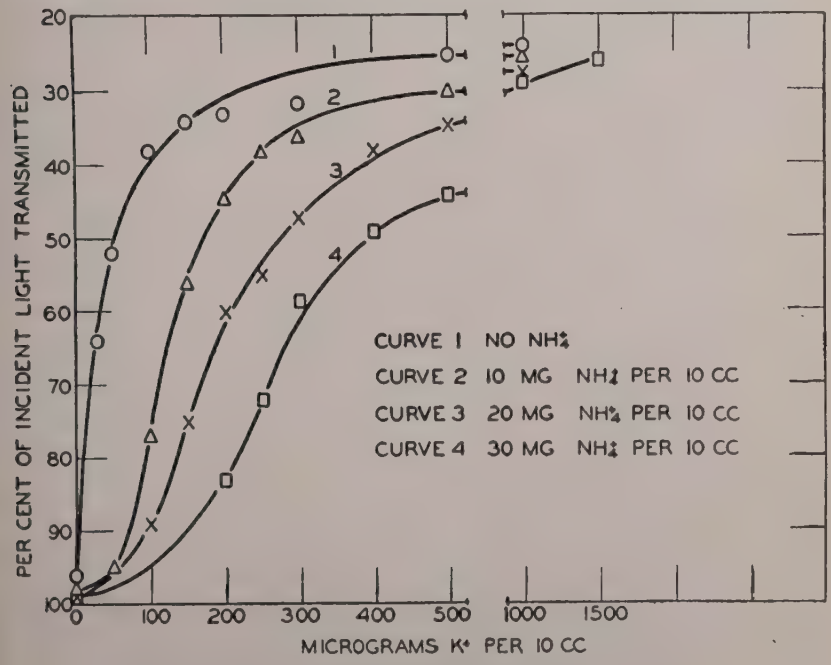

FIGURE 2. The effect of NH_4^+ on the response of *Lactobacillus casei* to K^+.

sented above, K^+ is the nutritionally essential ion, M_E, in the absence of which enzymatic processes essential for growth of the bacteria cannot proceed. At high concentrations, the structurally related Na^+ serves as the toxic ion, M_T, which by mass action produces an inactive metalloprotein unless more K^+ is added.

The ammonium ion, too, acts antagonistically to K^+ for *L. casei*, as shown in FIGURE 2. The molar ratios of NH_4^+ to K^+, at which half-maximum growth of several different organisms occurs, is shown in TABLE 2. Again, the constancy of these ratios at increasing concentrations of NH_4^+ indicates the competitive nature of the relationship between NH_4^+ and K^+ for these organisms, a relationship which is most easily explained in the same fashion discussed for Na^+ above. Comparison of these figures with those of TABLE 1 reveals that NH_4^+ is considerably more effective than Na^+ in counteracting the growth-promoting effects of K^+ for these organisms. For a widely different organism, *Saccharomyces carlsbergensis* 4228, for which the same

TABLE 2

MOLAR RATIOS OF NH_4^+ TO K^+ PERMITTING HALF-MAXIMUM GROWTH AT VARIOUS LEVELS OF ADDED NH_4^+

	mg. NH_4^+ per 10 cc. medium			
	10	*20*	*30*	*40*
	$[NH_4^+]/[K^+]$ for half-maximum growth			
L. arabinosus	115	135	149	140
L. casei	176	238	238	230
S. faecalis	88	105	112	115
L. mesenteroides 9135	230	268	280	208

TABLE 3

EFFECT OF Na^+ AND NH_4^+ ON THE RESPONSE OF *S. carlsbergensis* TO K^+

	mg. Na^+ per 10 cc.			*mg. NH_4^+ per 10 cc.*	
γK^+ per 10 cc.	*0*	*25*	*50*	*25*	*50*
	*% incident light transmitted**				
0	93	100	100	100	99
100	37	92	100	98	98
1000	15	25	79	26	42
10,000	15	16	27	17	22

* 24 hrs. incubation.

general relationships hold, Na^+ is more effective than NH_4^+ ion as an antagonist to K^+ (TABLE 3). It is interesting to note in passing that an ion which is a normal and important intermediate in cellular metabolism, such as NH_4^+, may serve in this way as an inhibitor of specific processes when present in sufficiently high amounts. It is quite possible that the amazing self-regulation of metabolism in living cells may in part be effected by control mechanisms such as this, which come automatically into play when certain products accumulate in excess.

An unusual case is presented by Rb^+. For *Streptococcus faecalis*, this element is the nutritional equivalent of K^+ (FIGURE 3). This must mean that Rb^+ can substitute for K^+ in all of the enzymic reactions essential for

growth of this organism in which K^+ is normally involved. Rb^+ similarly permits growth of *L. casei*, though not to the same high levels permitted by K^+ (FIGURE 4). For *Leuconostoc mesenteroides* 8042, however, no growth occurs when Rb^+ is added to a K^+-free medium (FIGURE 4). Now, several enzymatic reactions which require K^+ have been described, and it would be surprising indeed if Rb^+ replaced K^+ completely in each of these functions for an organism such as *S. faecalis*, and in none of them for an organism such as *L. mesenteroides*. For the latter organism, however, at least one of the essential roles of K^+ cannot be filled by Rb^+; otherwise growth would occur

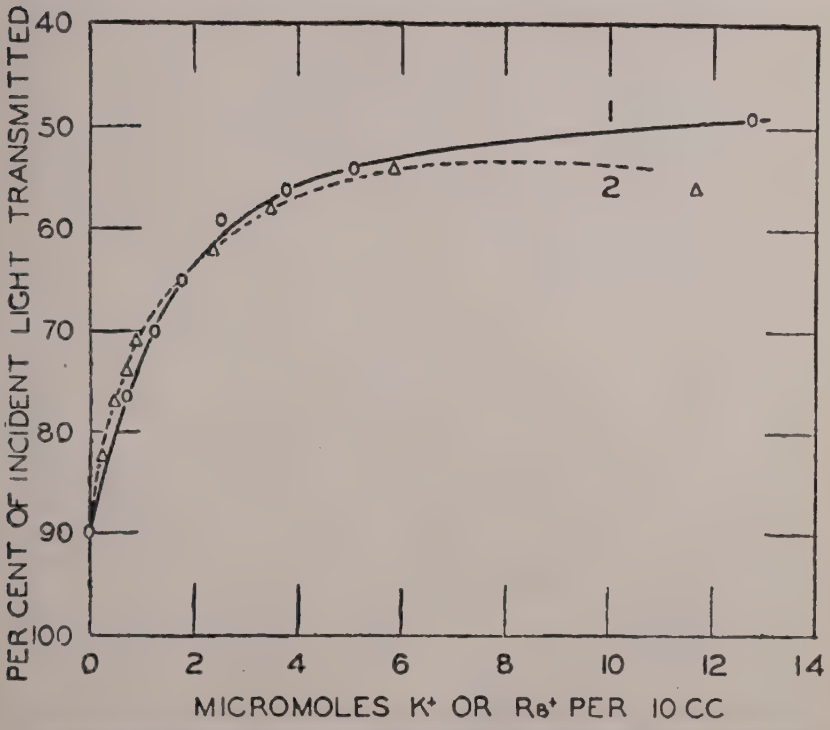

FIGURE 3. Comparative effects of Rb^+ and K^+ on growth of *Streptococcus faecalis*. Curve 1, K^+; Curve 2, Rb^+.

with Rb^+ alone. From the data of TABLE 4, it appears highly probable that some of the functions normally filled by K^+ in this organism can be filled by Rb^+, for the K^+ requirement is considerably reduced by culturing the organism in the presence of moderate amounts of Rb^+. At higher concentrations, however, Rb^+ (like Na^+ and NH_4^+) becomes "toxic" for this organism, and this inhibitory effect again is alleviated by added K^+ in a competitive manner (TABLE 5).

The observation that the same metallic ion, Rb^+, completely replaces K^+ for one organism, does so only partially for another, and, at high concentrations, is antagonistic to K^+ for the latter organism provides excellent evi-

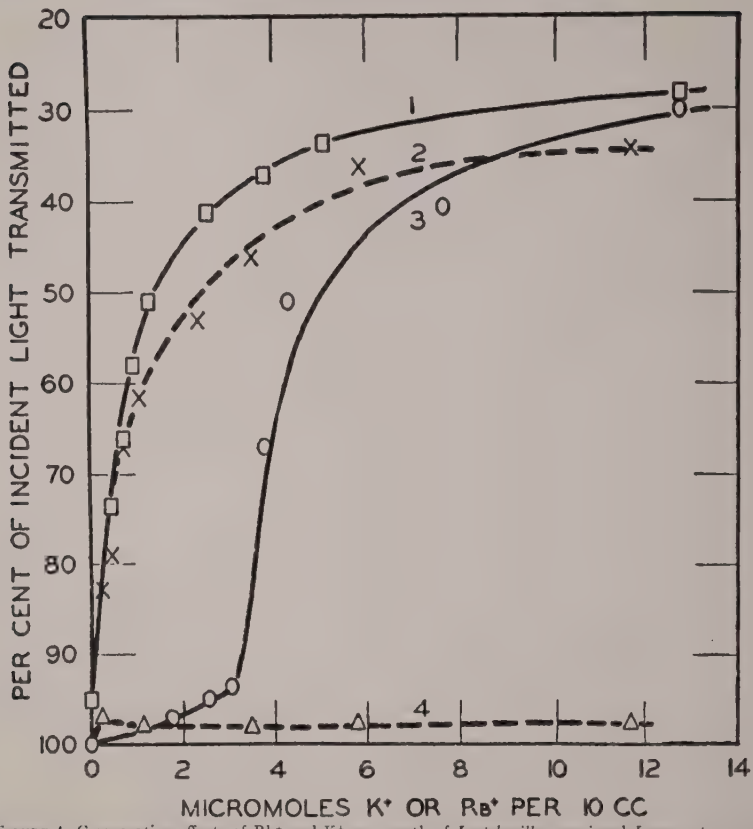

FIGURE 4. Comparative effects of Rb^+ and K^+ on growth of *Lactobacillus casei* and *Leuconostoc mesenteroides* 8042. Curves 1 and 2, response of *L. casei* to K^+ and Rb^+, respectively. Curves 3 and 4, response of *L. mesenteroides* to K^+ and Rb^+, respectively.

TABLE 4

SPARING ACTION OF Rb^+ ON THE K^+ REQUIREMENT OF *L. mesenteroides* 8042

γ K^+ per 10 cc.	γ Rb^+ per 10 cc.	
	0	300
	% incident light transmitted*	
0	98	98
50	98	62
100	56	37
150	47	34
1000	26	25

* 24 hrs. incubation.

dence for the validity of the mechanism of antagonistic effects presented in this paper. For most enzymatic processes involving K^+, Rb^+ is so similar that it can fulfill the same function efficiently. Certain K^+-activated en-

zymes are adapted so specifically to this latter ion, however, that Rb^+ does not replace it. At high concentrations, however, some combination of Rb^+ with such enzymes would appear to occur, resulting in an inactive metalloprotein and consequent failure of growth. Such combination is readily reversible; hence addition of sufficient K^+ again permits growth of the organism. Na^+, NH_4^+, and Cs^+ [9] ions are less similar to the K^+ ion. They do not permit growth of any of these organisms in the absence of K^+, but they retain the capacity to combine with certain proteins of the organism which normally bind K^+ and thereby to act as competitive antagonists to the K^+ ion.

TABLE 5
EFFECT OF K^+ ON THE INHIBITORY ACTION OF Rb^+ FOR *L. mesenteroides* 8042

γK^+ per 10 cc.	mg. Rb^+ per 10 cc.			
	0	10	20	30
	% incident light transmitted			
0	97	90	93	93
500	29	60	84	89
1000	28	45	64	84
20,000	30	30	30	31

TABLE 6
EFFECT OF Rb^+ ON THE INHIBITORY ACTION OF NH_4^+ FOR *L. casei*

γRb^+ per 10 cc.	mg. NH_4^+ per 10 cc.		
	0	20	40
	% incident light transmitted		
0	67	89	90
100	46	78	89
500	29	51	66
10,000	33	24	25

If Rb^+ is a fairly complete substitute for K^+ in growth of *S. faecalis* and *L. casei*, as is indicated by its equivalent growth-promoting power (FIGURES 3 and 4), then it should act like K^+ in alleviating the inhibitory action of Na^+ and NH_4^+ for these organisms. TABLE 6 shows that the inhibitory action of NH_4^+ for *L. casei* is alleviated by Rb^+, just as it is by K^+. Similar data show that Rb^+ will also counteract the toxic effects of Na^+ for this organism. This is further evidence that Rb^+, K^+, Na^+, and NH_4^+ have the same locus of action within these cells.

The Effect of Ions Related to Mn^{++} (cf. Ref. 10). *Lactobacillus arabinosus* grows well in a medium composed of pure amino acids, glucose, vitamins, and appropriate buffers and mineral salts. Suitable deletion experiments

show that K^+ and Mn^{++} are the only metallic ions which must be added to such a medium to permit growth. Even pretreatment procedures involving successive absorptions of the medium with the growing organism[10, 11] fail to reveal any requirement for additional metallic ions, although it is still possible that very small amounts of such additional ions are necessary and are supplied as contaminants with the various ingredients of the basal medium.

TABLE 7
REVERSAL OF Zn^{++} TOXICITY FOR *L. arabinosus* BY Mn^{++}

γMn^{++} per 10 cc.	Zn^{++} per 10 cc.			
	0		400	
	% incident light transmitted			
	24 hrs.	45 hrs.	24 hrs.	45 hrs.
0	95	95	100	99
1	76	70	—	—
10	37	33	—	—
100	20	19	99	99
200	—	—	99	22
300	—	—	97	19
400	—	—	27	18

TABLE 8
REVERSAL OF Zn^{++} TOXICITY FOR *L. arabinosus* BY Mg^{++}, Ca^{++}, AND Sr^{++}

γMg^{++} Ca^{++} or Sr^{++} per 10 cc.	No Mn^{++} or Zn^{++}			4γ Mn^{++} + 400γ Zn^{++} per 10 cc.*		
	% incident light transmitted†					
	Mg^{++}	Ca^{++}	Sr^{++}	Mg^{++}	Ca^{++}	Sr^{++}
0	95	95	97	100	100	98
50	—	—	—	—	55	—
100	92	95	97	98	50	99
300	—	—	—	—	48	55
400	92	95	97	61	46	52
500	91	96	98	43	46	55

* 4 γ per 10 cc. is less than the Mn^{++} requirement of *L. arabinosus* for maximum growth on this medium The amount of growth attainable upon complete reversal of the Zn^{++} toxicity is thus limited by the Mn^{++} concentration.
† 24 hrs. incubation.

If, in such a medium, K^+ is supplied in excess but Mn^{++} is added in only small amounts, relatively low concentrations of Zn^{++} are found to inhibit growth. Such inhibitory effects are prevented completely (at moderate levels of Zn^{++}) if enough Mn^{++} is added (TABLE 7). Thus, an antagonistic relationship exists between Zn^{++} and the nutritionally essential ions, Mn^{++}, and it is attractive to picture the inhibition by Zn^{++} and its reversal by the same general mechanism discussed earlier.

In apparent conflict with this simple explanation, however, is the observation that, in the presence of concentrations of Mn^{++} insufficient to overcome the inhibitory effect of Zn^{++}, three other ions, Mg^{++}, Ca^{++}, and Sr^{++}, also prevent the "toxic" action of Zn^{++} (TABLE 8). Of the three, Ca^{++} is most

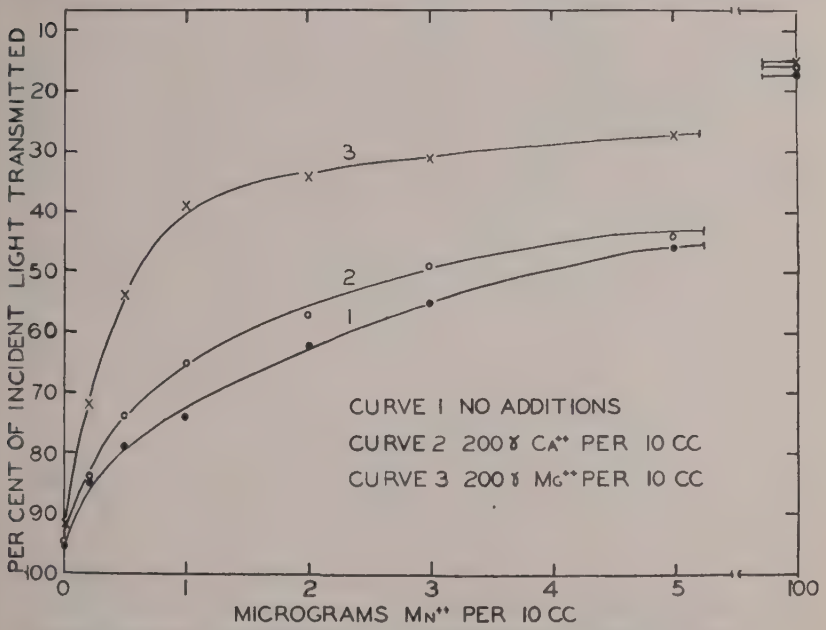

FIGURE 5. The sparing action of Mg^{++} and Ca^{++} on the requirement of *L. arabinosus* for Mn^{++}.

TABLE 9

SPARING ACTION OF Sr^{++} ON THE Mn^{++} REQUIREMENT OF *L. arabinosus*

Mn^{++} γ per 10 cc.	*No Sr^{++}*	*200 γSr^{++} per 10 cc.*
	*% incident light transmitted**	
0	98	97
2	62	58
5	51	44
10	37	33
100	17	18

* 24 hrs. incubation.

effective and is, in fact, more effective than Mn^{++} (*cf.* TABLE 7). This fact, and the fact that no growth occurs in the presence of any of these ions if Mn^{++} is omitted from the medium, eliminates the possibility that the ability of these ions to reverse Zn^{++} inhibition results from contaminating traces of Mn^{++} in these added salts or in the medium.

An explanation of these results consistent with our theory that such

antagonistic actions have a nutritional basis is, however, possible. It was noted earlier that a "foreign" ion, Rb^+, can replace K^+ completely for growth of some organisms and partially replace it for others. Furthermore, for organisms in which it is the nutritional equivalent of K^+, Rb^+ prevents the toxic action of Na^+ and NH_4^+, which are antagonistic to K^+. In the present instance, neither Mg^{++}, Ca^{++}, nor Sr^{++} is the *complete* nutritional equivalent of Mn^{++}, *i.e.*, none of them replaces it for growth. Several enzymes have been described, however, which are activated by Mn^{++} and, of these, several (*e.g.*, arginase[12] and others[13]) can also be activated by other divalent metal ions. It thus is logical to assume that in *L. arabinosus* there are some enzymatic processes for which Mn^{++} is a specific activator and some for which Mn^{++} can function, but for which certain other divalent metal ions, among them Mg^{++}, Ca^{++}, and Sr^{++}, also can function. If the toxic action of Zn^{++} resulted from its combination with one of these latter enzymes, it would be expected that Ca^{++}, Sr^{++}, or Mg^{++} might reverse this toxic effect as well as Mn^{++}, even though they are unable to substitute for Mn^{++} in *all* of its multiple functions.

If this is a true explanation, and Mg^{++}, Ca^{++}, and Sr^{++} are able to function in place of Mn^{++} with certain of the enzymes of *L. arabinosus*, one would expect that, even though these ions were unable to support growth in the absence of Mn^{++}, the requirement for Mn^{++} should be decreased in their presence. That this is true is shown for Mg^{++} and Ca^{++} in FIGURE 5 and for Sr^{++} in TABLE 9. In these experiments, the Mn^{++} requirement was determined in the presence of an excess of each of these ions. Mg^{++} shows a greater sparing action on the Mn^{++} requirement than does Ca^{++}, which is in turn more effective than Sr^{++}. We should interpret this to mean that several of the enzymes of *L. arabinosus* can be activated by either Mn^{++} or Mg^{++}, and fewer still by either Mn^{++}, Mg^{++}, Ca^{++}, or Sr^{++}. It is presumably with one of the latter group that Zn^{++} may combine to form a catalytically inactive metalloprotein.

These findings and interpretations are of importance also to those interested in determining the inorganic requirements of bacteria and other living organisms. With *L. arabinosus*, we have positive evidence of a requirement for only two metallic ions, K^+ and Mn^{++}. It is clear from FIGURE 5, however, that Mg^{++} and Ca^{++}, if present, may also be used by this organism for certain functions which Mn^{++} may also serve. It is meaningless to inquire which ion the organism "prefers" to use, since this will obviously depend upon the relative concentrations of the various ions in its nutritive environment. If the reasoning outlined above is correct, however, it is apparent that Ca^{++} has a greater "affinity" for certain of these enzymes than has either Mn^{++} or Mg^{++}, as judged by their relative activities in preventing Zn^{++} toxicity. Thus, those inorganic ions which are the sole ones essential for growth of an organism under minimal nutritive conditions may not be the only ones (or even the principal ones) used for metabolic purposes under conditions of practical nutrition. Stated differently, the fact that a given inorganic ion is not essential for growth under a given set of conditions does

not discount the possibility that it may be useful in metabolism or that it may even become essential under another set of conditions.

In summary, several instances of ion antagonism in the lactic acid bacteria have been discussed which are most readily explained on nutritional grounds. According to the views developed, certain metallic ions which inhibit growth may be looked upon as structural analogues of other metallic ions which are essential for certain metabolic processes involved in growth (*e.g.*, in the activation of various cellular enzymes). At appropriate concentrations, competition between the essential ion and its inhibitory analogue for combination with an essential enzyme will occur. Depending upon the relative concentrations of antagonistic ions, a catalytically active metalloprotein results, permitting growth; or a catalytically inactive metalloprotein results, with consequent growth inhibition. Because many metal-activated enzymes are relatively nonspecific in so far as the cation required for activation is concerned, several cations may reverse the inhibitory action of a single inhibitory ion. In some instances, such effective cations may not appear essential for growth even though they play an important and useful role in the normal metabolism of the cell.

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INTERFERENCE WITH HORMONAL EFFECTS BY ANTIVITAMINS AND COMPETITION BETWEEN STRUCTURALLY SIMILAR STEROID HORMONES

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The formation of new tissue in the male and female genital tract under the influence of the gonadal steroid hormones has been the subject of extended study by endocrinologists. Relatively little attention has been given, however, to the nutritional aspects of this special form of tissue deposition.

In this paper, I shall briefly describe the growth responses which are obtained in the secondary sex organs when either estrogens or androgens are administered to sexually immature or previously gonadectomized animals which are maintained on a complete diet. Let us consider, for example, the responses observed in the oviduct of the two-week-old New Hampshire Red chick treated with a maximally effective dose of diethylstilbestrol on each of six successive days. FIGURE 1 shows the comparative size and condition of the oviduct of a chick so treated and its untreated control. The increment in tissue mass is approximately forty-fold, that is, from a weight of about 20 mg. to that of 800 to 900 mg. Dry weight determinations and histological examination indicate that in the main this is true tissue growth.

If we vary the dose of estrogen administered or the time during which any stated dose is applied, it becomes apparent that we are dealing with a quantitatively controlled growth response, possessing a characteristic latency and progression in time. It is also clear that the response reaches a distinct quantitative plateau which can not be exceeded. In many respects, then, we are dealing with a rapid anabolic process which has many features in common with the type of microbiological growth process with which the reader is perhaps more familiar.

FIGURE 2, taken from Lauson *et al.*,[1] shows a similar growth effect of estradiol on the uterus of the sexually immature rat. FIGURE 3 presents the uterus of a sexually immature ovariectomized rat treated for 48 hours with a maximally effective dose of estradiol in contrast with the uterus of an untreated control animal. There are numerous examples of such quantitatively controllable responses to various histotrophic hormones, but one additional example will prove pertinent to our subsequent discussion. When castrated male rats are treated with varying doses of testosterone propionate for variable periods of time, a similar type of rapid but highly regulated growth response is seen both in seminal vesicles[2] and in the prostate gland (FIGURE 4).

We have been investigating the role of certain dietary factors in these various types of hormone-induced tissue growth and we will now turn to a somewhat detailed consideration of one of these studies.

Working with the New Hampshire Red female chick maintained on a synthetic diet of the composition shown in TABLE 1, we found that the ex-

pected tissue-growth response to a maximally effective dose of estrogen was markedly reduced. Instead of the expected forty-fold increment, we would observe only a five-fold enlargement of the oviduct. We soon found that concentrates of liver, yeast, and spinach would restore the tissue-growth effect to nearly the expected level.[3] Subsequently, as folic acid became identified as a distinct dietary factor, we could demonstrate that the addition

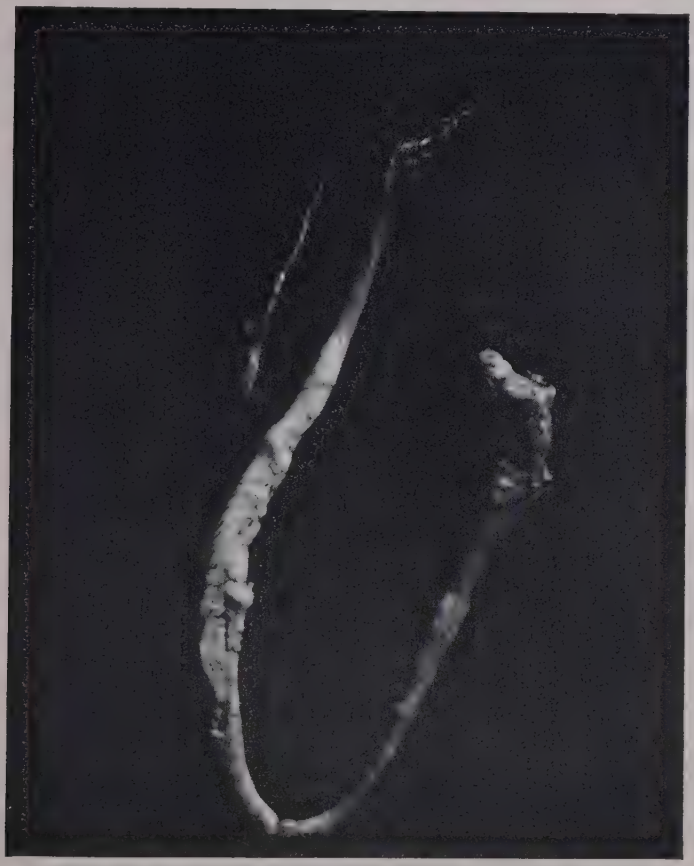

FIGURE 1. Chick oviducts (right) maximal effective dose of estrogen; (left) untreated.

of crystalline pteroylglutamic acid to our synthetic diet would result in nearly optimal growth effects in the oviduct. Moreover, the total response obtained varied with the dose of folic acid fed. It was therefore apparent that hormone-induced tissue growth may under some conditions reflect the dietary intake of specific nutrilites.

As antagonists to folic acid became available, we undertook the study of their effects upon the estrogen response in the chick and rat.

Methods and Materials

(*A*) *Chicks.* New Hampshire Red chicks of the same flock were used throughout. They were received the day after hatching and were maintained in electrically heated brooders. No food was given, but tap water was provided *ad libitum.* On each of the first three days in the laboratory, the chicks were injected subcutaneously with from 0.2 cc. to 1.0 cc. of an

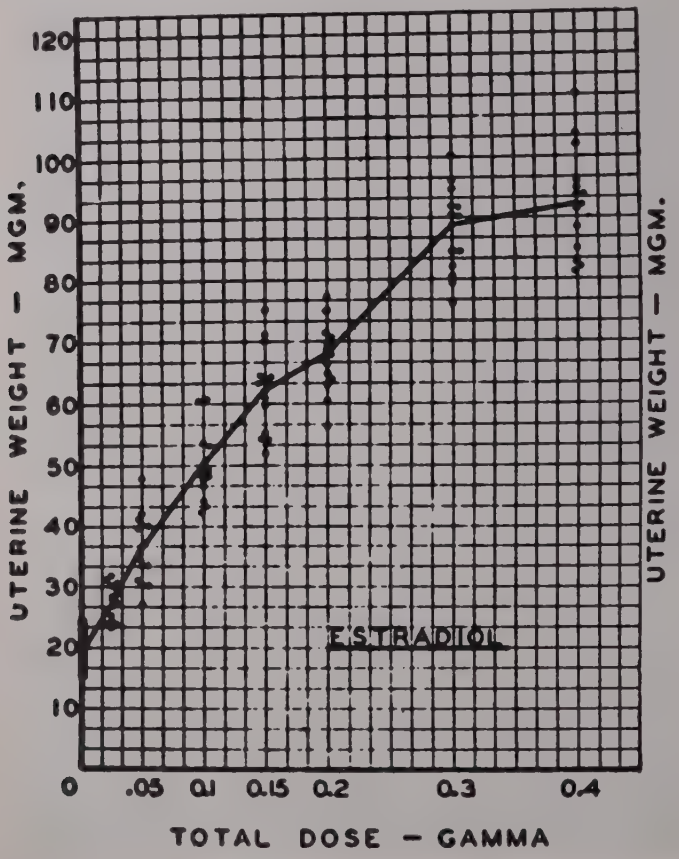

FIGURE 2. Growth effect of estradiol on the uterus of the sexually immature rat (from Lauson *et al.*[4]).

aqueous solution or suspension of the folic acid analogue to be tested. In experiments designed to show the reversibility of the observed inhibition, the chicks also received subcutaneously each day 5 mg. of folic acid dissolved in 1 cc. of .05/N NaOH. The folic acid was always injected one hour before the administration of the analogue. On each of the last two days of the test, 1 mg. of diethylstilbestrol dissolved in 0.2 cc. of corn oil was injected subcutaneously. Twenty-four hours after the last injection, the animals were autopsied. The body weight was recorded and the genital tract dissected

FIGURE 3. Uteri of immature ovariectomized rats: untreated controls (above); estradiol, maximal effective dose—48 hr. test (below).

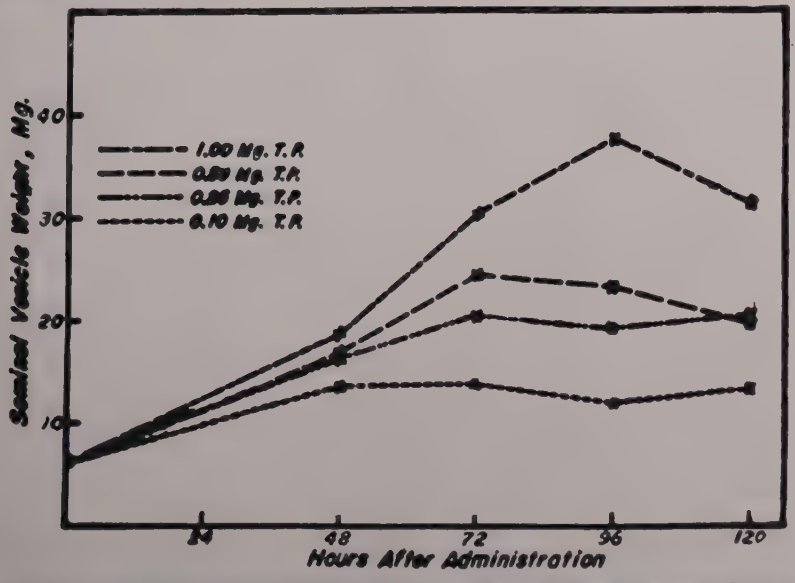

FIGURE 4. (From Hays and Mathieson.[2]) The response of seminal vesicles in castrate male rats to various doses of testosterone propionate at 48, 72, 96, and 120 hours. The average seminal vesicle weight of castrate controls was 6.1 mg.

out and weighed. Control groups of animals receiving diethylstilbestrol alone and those receiving no treatment were autopsied at the same time.

(*B*) *Rats.* Twenty-one-day-old weanling female rats of the Wistar Strain were ovariectomized and given a pulverized stock ration supplemented with 2 per cent dried liver *ad libitum*. After a rest period of from 3 to 5 days, each rat was given the test dose of folic acid analogue subcutaneously in 0.2 cc. to 1 cc. of an aqueous solution or suspension on each of three consecutive days. On the second and third day of treatment, the rats also received 10 micrograms of estradiol in 0.5 cc. distilled H_2O subcutaneously. In the reversal experiments, the folic acid was administered subcutaneously in 1 cc. of .05/N NaOH at the rate of 5 mg. per day for 4 consecutive days,

TABLE 1
COMPOSITION OF PURIFIED CHICK DIET

Vitamin-free casein	25
Gelatin	10
l-Cystine	0.3
Choline chloride	0.2
Corn starch	52.4
Cellulose	3.0
Crisco	4.0
Salts	5.0
$MnSO_4$	0.1
Vit. D	160 U.S.P. Units
Vit. A	1600 U.S.P. Units
α-Tocopherol	28 mg.
Vit. K	5.0 mg.
Thiamine	0.4 mg.
Riboflavin	0.8 mg.
Pyridoxine	0.6 mg.
i-Inositol	50.0 mg.
p-Aminobenzoic acid	15.0 mg.
Niacin	2.0
Calcium pentothenate	1.1 mg.
Biotin	10.0 μg.

starting the day preceding treatment with the analogue. At autopsy, 24 hours after the last injection, the uteri were dissected out, freed of distending fluid, and weighed to the nearest milligram.

The folic acid employed was crystalline pteroylglutamic acid. The analogues investigated included:* (a) 4-aminopteroylglutamic acid; (b) 4-aminopteroylaspartic acid; (c) 4-amino-N^{10}-methyl pteroylglutamic acid; (d) 2,4-diamino-6,7-dimethyl pteridine; (e) 2-amino-4 hydroxy-6,7-di(*p*-amino-phenyl) pteridine; (f) 2,4-diamino-6,7-diphenyl pteridine; (g) 4-desoxypteroyglutamic acid; and (h) 2-amino-4-hydroxy-6,7-diphenyl pteridine.

FIGURES 5 and 6 present representative data obtained with aminopterin on the chick and the rat. It will be seen that the antagonist exerts a quantitative inhibitory effect upon the tissue growth response to an otherwise

* (a), (b), (c), and crystalline folic acid kindly supplied by the Lederle Laboratories, Pearl River, New York; (d), (e), and (f) kindly supplied by Dr. R. A. Brown, Parke Davis & Co., Detroit, Michigan; and (g) and (h) kindly furnished by Dr. C. K. Cain, Cornell University, Ithaca, New York.

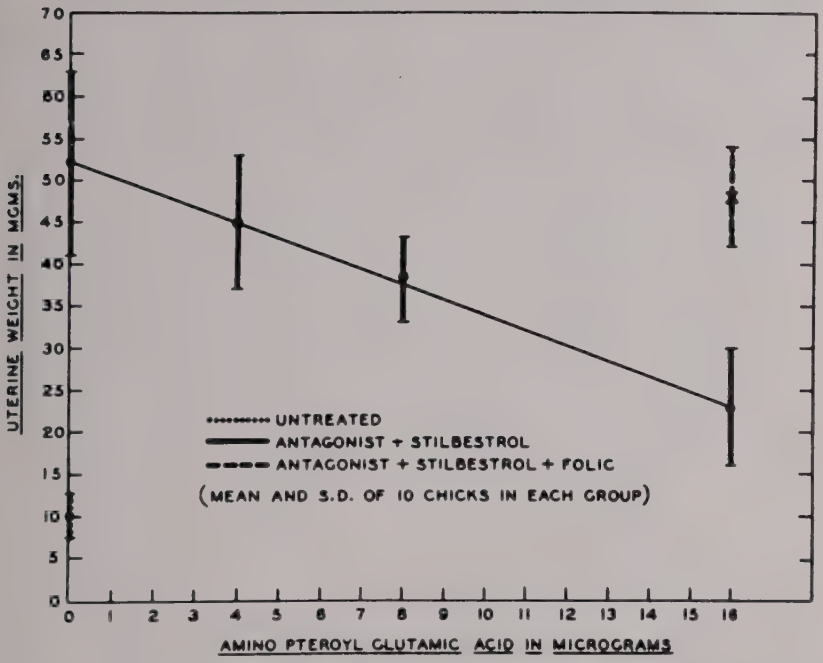

FIGURE 5. Effect of folic antagonist on chick oviduct response to stilbestrol.

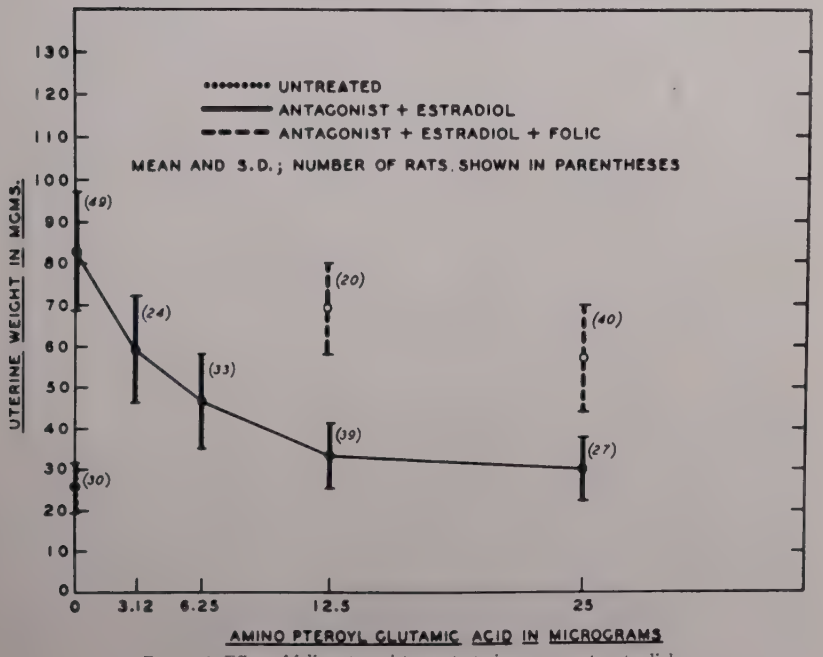

FIGURE 6. Effect of folic antagonist on rat uterine response to estradiol.

maximally effective dose of estrogen. Moreover, this inhibition is almost completely prevented by the administration of very high doses of folic acid.

Similar observations made in the chick with other folic antagonists are presented in TABLE 2. The pteridine compounds proved essentially inactive in the limited doses employed, whereas 4-amino-pteroylaspartic acid and

TABLE 2

EFFECT OF FOLIC ACID ANTAGONISTS ON ESTROGEN RESPONSE IN CHICK OVIDUCT*

Antagonist	*Daily dose, mg.*	*Oviduct weight— mg.*
4-amino-N^{10}-methyl pteroylglutamic acid	0.2	23 ± 5
	1.0	23 ± 3
	0.2†	57 ± 8
	0.0	51 ± 6
4-aminopteroylaspartic acid	0.2	48 ± 5
	0.4	44 ± 5
	0.8	33 ± 9
	2.0	29 ± 6
	3.0	22 ± 7
	4.0	20 ± 3
	0.0	54 ± 8
4-desoxypteroylglutamic acid	1.0	50 ± 7
	5.0	55 ± 6
	25.0	47 ± 6
	50.0	23 ± 4
	0.0	59 ± 5
2,4-diamino-6,7 dimethyl pteridine	1.0	48 ± 5
	5.0	58 ± 12
2-amino-4-hydroxy-6,7-diphenyl pteridine	1.0	50 ± 4
	5.0	53 ± 5
	9.0	47 ± 4
2-amino-4-hydroxy-6,7-di(*p*-aminophenyl) pteridine	1.0	48 ± 6
	5.0	62 ± 11
2,4-diamino-6,7-diphenyl pteridine	1.0	52 ± 4
	5.0	51 ± 8
	9.0	52 ± 9

* All animals treated as described in text; 10 chicks in each group.
† Also given 5 mg. folic acid daily.

4-amino-N^{10}-methyl folic acid were found to be active. Limited activity was observed for 4-desoxy-pteroylglutamic acid.

That the relationship between folic acid and aminopterin in regulating the quantitative growth response to estrogen represents a truly quantitative antagonism is shown by the data presented in FIGURE 7. In these experiments, the chicks were first depleted by having been fed for 16 days on a folic-deficient diet. Then they were treated for 5 days with a high dose of stilbestrol and with the indicated dose of folic acid or with the stated combination of folic acid antagonist. It is clear that on a fixed dose of folic acid

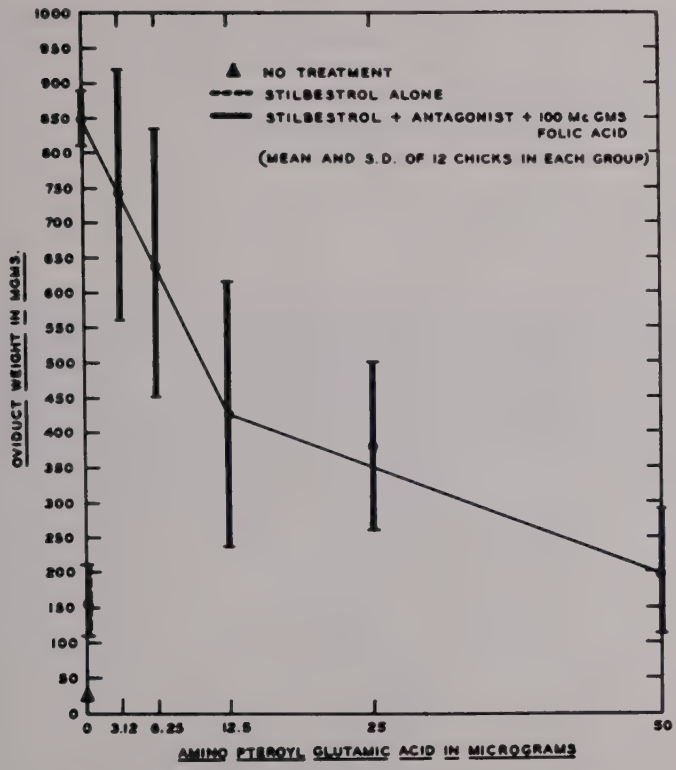

FIGURE 7. Quantitative antagonism between folic acid and aminopterin?

TABLE 3

EFFECT OF 2,6-DIAMINOPURINE ON ESTROGEN RESPONSE IN CHICK GENITAL TRACT*

Group	*Stilbestrol*	*2,6-Diamino purine (mg.)*	*Adenine sulfate (mg.)*	*Number of chicks*	*Oviduct weight (mg.)*
I	−	—	—	10	8 ± 3
II	+	—	—	10	50 ± 5
III	+	15	—	10	14 ± 5
IV	+	10	—	10	22 ± 3
V	+	10	4	10	30 ± 5
VI	+	10	8	10	37 ± 6
VII	+	10	50	8	42 ± 4

* Day-old N. H. red chicks were used; they were given no food but water *ad libitum*. Diethylstilbestrol given at 1 mg. daily subcutaneously in 0.2 cc. corn oil for 2 days. Other compounds injected at indicated daily dose for 3 days in 1.0 cc. aqueous solution or suspension, except for group VII which received adenine by capsule.

the degree of inhibition obtained varies with the dose of added inhibitor. It is to be emphasized that these phenomena are observed in the presence of a maximally effective dose of estrogen.

These data permit us also to calculate roughly an inhibitory ratio for aminopterin. According to these data, one part of aminopterin will inhibit about 2 parts of folic acid, a ratio somewhat comparable to that derived from microbiological data.

Dr. G. Hitchings has recently made available to us a number of purine and pyrimidine analogs, which are described in detail elsewhere in this monograph.* In view of the ability of some of these compounds to inhibit the growth of folic acid-requiring organisms, we have tested them for their effect upon estrogen-induced tissue growth, employing the short chick test already

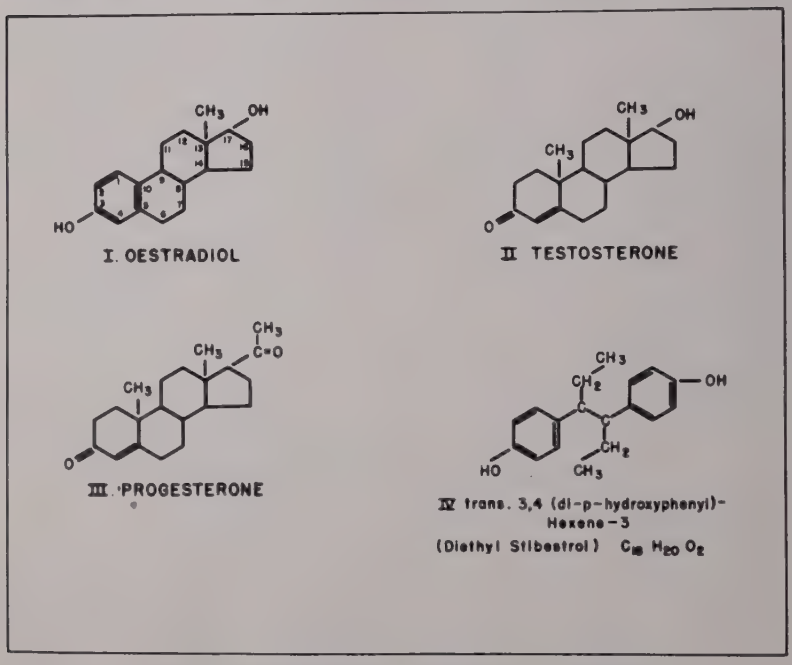

FIGURE 8. Structural formulas of steroid hormones and stilbestrol

described.[4, 5] One of these derivatives, 2-6 diaminopurine, has been found to exert a marked inhibitory effect and this inhibition is largely reversed by adenine, *i.e.*, 6-aminopurine (TABLE 3).

Our experience with these remarkable inhibitory effects of the antimetabolites has formulated for us a similar approach to a separate but closely related phenomenon, namely, the marked biological antagonism between several chemically related steroid hormones. Although these opposing biological effects have received considerable experimental and clinical attention, little effort has been made to rationalize what has been observed. We have previously suggested that the Woods-Fildes principle of competition between chemically similar metabolites may apply.[6] Dr. D. W. Woolley has

* Pp. 1318. We wish to acknowledge with gratitude the provision of these purine antagonists by Dr. Hitchings (Wellcome Research Laboratories, Tuckahoe, N. Y.).

also touched on this point in his writings.[7] It may therefore prove profitable to have a few selected examples of the experimental data supporting and opposing such a view briefly outlined for your consideration.

Let us first discuss the interesting antagonism observed between progesterone and several estrogenic compounds. The chemical relationship between these highly active hormones is shown in FIGURE 8. In FIGURE 9 are represented the tissue growth effects observed in the oviduct of the chick treated with a maximally effective dose of estrogen as compared with the markedly reduced response obtained when the estrogen is combined with

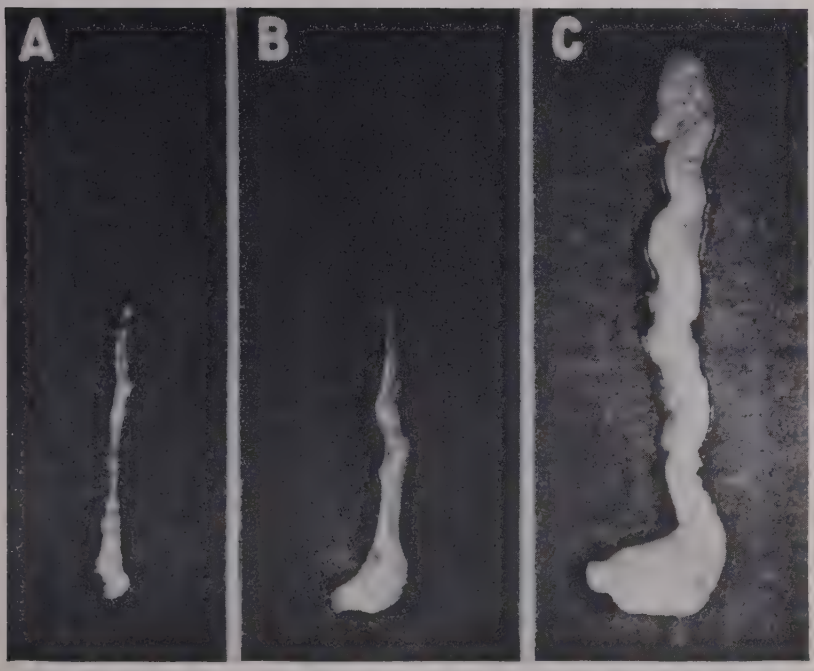

FIGURE 9. A, oviduct weighing 23 mg. from untreated chick; B, oviduct weighing 230 mg. from chick given 0.5 mg. progesterone plus 0.25 mg. stilbestrol in oil subcutaneously daily for 8 days; C, oviduct weighing 970 mg. from chick given 0.25 mg. stilbestrol in oil subcutaneously daily for 8 days.

progesterone. If one graphs the results obtained with increasing doses of progesterone in the presence of a maximally effective dose of stilbestrol (or estradiol) (FIGURE 10), one sees that there is a distinct relationship between inhibitory effect and progesterone dosage over a considerable range. Beyond this range the inhibitory effect levels off. We do not have available the necessary data to characterize fully this particular example of estrogen-progesterone antagonism. However, the observations of Leonard *et al.*[8] on the effect of varying combinations of estrogen and progesterone on the rabbit uterus and of Courrier and Cohen-Solal,[9] employing the vaginal mucosa of the castrate rat, indicate that the antagonism is reciprocal. The qualitative nature of the end point employed by Leonard *et al.*, namely the progesta-

tional transformation of the endometrium, must be considered in evaluating these data. Courrier[10] calculated, however, that one part of estradiol would neutralize the progestational effect of 40 parts of progesterone.

Lipschutz and Iglesias[11] have shown that prolonged administration of estrogens will induce fibroid tumors in the uteri of guinea pigs. This fibromatogenic effect can be completely prevented by the simultaneous administration of either progesterone or several chemically related steroids.[12, 13] Lipschutz emphasizes the fact that this "anti-tumorigenic" action cannot be correlated with any other known biological property of the effective steroids.

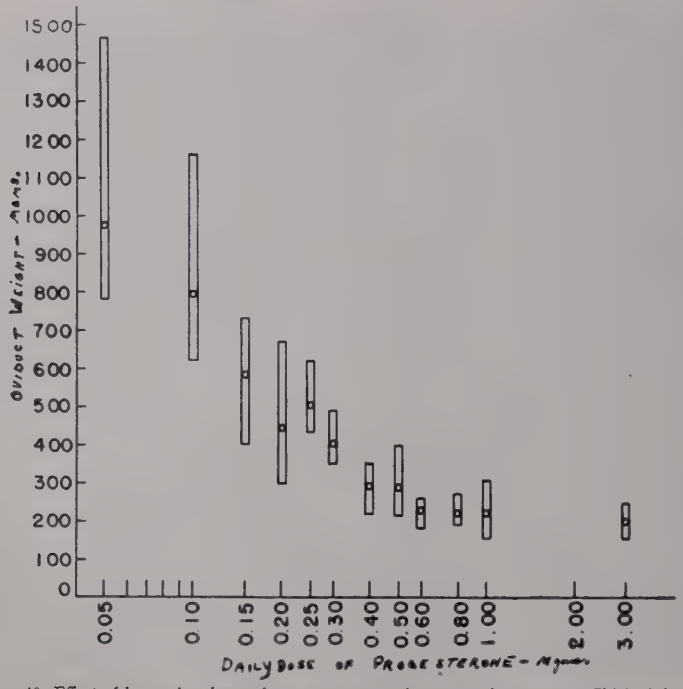

FIGURE 10. Effect of increasing doses of progesterone on tissue-growth response. Chicks injected with 0.26 mg. stilbestrol and indicated dosage of progesterone in oil daily subcutaneously for 8 days.

He adds that "there is place for the hope that anti-tumorigenic steroids having no other pharmacological action may be obtained by chemical synthesis".[14]

A second example worthy of our consideration is the demonstrated antagonism between the male and female sex hormones, *i.e.*, between estrogen and androgen. Only a few illustrative observations from the great mass of experimental and clinical data relating to this antagonism will be cited. Rapid growth may be induced in the comb of the chick by androgen administration (FIGURE 11). Gley and Dolor[15] and subsequently Muhlbock[16] have shown that the simultaneous administration of estradiol will quantitatively repress this androgen-induced growth effect in the capon comb.

Huggins and Clark[17] demonstrated a measurable increment in secretion of fluid from the prostate gland of the castrated dog in response to testosterone

FIGURE 11. Androgen-induced growth effect in chick comb. Androgen stimulated comb (right); untreated control (left).

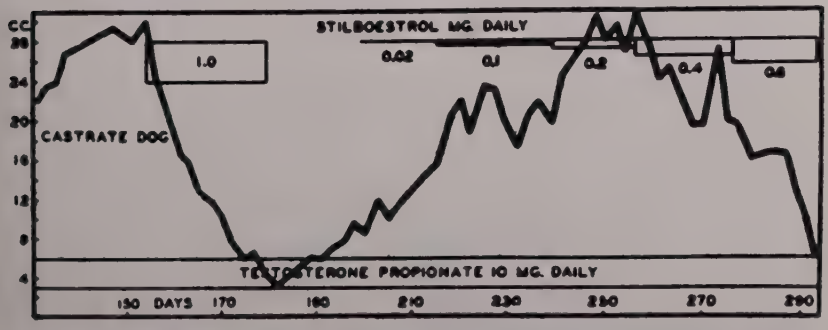

FIGURE 12 (from Huggins and Clark[17]). A castrate immature dog had been injected daily with tessosterone propionate, 10 mg. for 160 days, during which time the secretion had risen from 0.1 to 30.2 cc. This androgen was continued at the same rate throughout the experiment and in addition stilbestrol injections were begun. Daily amounts of stilbestrol, 1.0 mg. and 0.6 mg. caused a great decrease of secretion, while dosages of 0.02, 0.1, and 0.2 mg. did not affect the output; stilbestrol 0.4 caused a leveling of the rate of secretion (dog 5–10).

propionate administration. This secretory flow could be partly arrested by the simultaneous administration of small doses of stilbestrol and completely

arrested by larger doses (FIGURE 12). These and related data have led to the effective clinical management of cancer arising in the prostate gland by administration of antiandrogenic estrogens and/or castration. A quantitatively assessable feature of this disease in some cases is an elevation of the acid phosphatase level in the serum, the production of this enzyme being dependent upon androgen production. Huggins[18] reports that the serum acid phosphatase level may be either lowered by estrogen administration or elevated by androgen treatment. These observations present a striking example of a reciprocal, quantitative hormone antagonism in man.

We have already indicated that these biological antagonisms to progesterone and testosterone are observed with estrogens of the stilbene type as well as with those of true steroid structure. At first glance, this would seem to argue against the hypothesis that the observed effects are attributable to chemical similarity. Scheuler[19] has pointed out, however, that the stilbene molecule has a basic physico-chemical similarity to estrone with respect to certain molecular dimensions. It is this common property which he considers responsible for the estrogenic effect. We could consider also that this common feature serves as a basis for the antagonistic biological effects under discussion. Roblin[20] has presented similar physico-chemical considerations with respect to other antagonists.

It is fully appreciated that the quantitative data at hand are insufficient to permit an adequate characterization of the mechanisms involved. Moreover, what may appear superficially to be a quantitative interference may, on closer study, prove to be a qualitative alteration in the reacting tissue which limits the quantitative response. Nevertheless, it is felt that the tentative view expressed here that we may be dealing with competitive interference by chemically similar metabolites may serve some purpose. It may serve (a) to stimulate further synthesis and testing of biologically inert steroids and stilbenes having potential inhibitory properties and (b) to further quantitative study on the mechanisms involved in hormone antagonisms.

Summary

The tissue growth response to estrogen in the female chick and rat is quantitatively dependent upon folic acid. Accordingly, effective folic acid antagonists quantitatively interfere with this response and this interference is reversed by folic acid. A purine analogue (2,6 diaminopurine) will similarly inhibit the estrogen response in the chick and this inhibition is reversible by adenine. Thus, a hormone-induced tissue growth can be inhibited by effectively interfering with the biological action of a dietary trace factor.

A review of some of the features of the antagonism between (a) estrogen and progesterone and (b) estrogen and androgen suggests the tentative view that the principle of competition between chemically similar metabolites may in part account for some of the phenomena.

Our further comprehension of these phenomena may permit the development of chemical agents of therapeutic value in such conditions as prostatic and breast cancer in which a reduction of the biological effectiveness of endogenous steroid hormones has proven beneficial.[21, 22]

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THE STRUCTURAL BASES OF SOME AMINO ACID ANTAGONISTS AND THEIR MICROBIOLOGICAL PROPERTIES*

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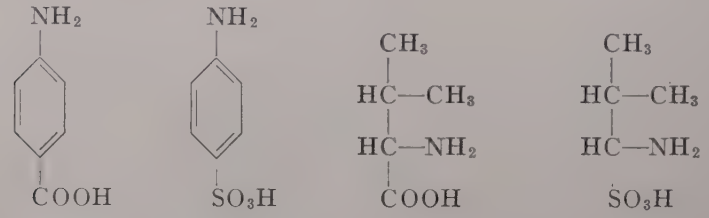
An intensive search for antagonists of most of the known metabolites began in 1940 after Woods[1] announced that the chemotherapeutic properties of sulfanilamide were due to competition with the structurally related metabolite, *p*-aminobenzoic acid. In only a few years, antagonists were known of most of the water soluble vitamins, of some hormones, some amino acids, and of other metabolites. The various analogues, their syntheses, and properties have been amply reviewed by Woolley,[2] McIlwain,[3] Roblin,[4] and others.

From the list of the known metabolite antagonists, several generally applicable structural changes can be selected: changes of the molecular structures which produced inhibitors of several vitamins. Sometimes these changes were effective in more than one general group of biocompounds, *e.g.*, vitamins and amino acids, and sometimes a structural change had been effected in only a single compound. Out of these different changes, however, came an incomplete and small catalog of effective structural alterations. Some of the structural changes which are the bases for the formation of antivitamins are listed in TABLE 1.

Some of these structural modifications were applied to other metabolites. Some were applied to amino acids. Many, however, have not yet been incorporated into enough different compounds to ascertain their general applicability. The different structures of the known amino acids still offer many more opportunities to test some of these indicated bases for the synthesis of metabolite inhibitors.



In this paper, a number of such modifications of amino acid molecules will be described.

* Much of the work reported in this paper was supported in part under contract with the Office of Naval Research.
† The author gratefully acknowledges the valuable support and assistance received from his associates throughout this work.

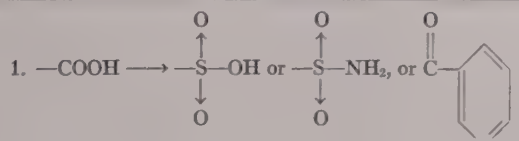
Sulfonic Acid Analogues of Amino Acids. Soon after it was known that sulfanilamide antagonized the utilization of PAB, McIlwain[7, 10] studied the bacteriostatic properties of several aminoalkyl sulfonic acids which are related to the corresponding amino acids in the same way that sulfanilic acid is related to PAB.

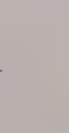
This same change has been introduced into other compounds, giving rise to analogues of glycine,[5–8*] alanine,[7–9] valine,[6, 7, 9, 10] leucine,[7, 9, 10] aspartic acid,[7, 10, 11] and phenylalanine.[9] Most of these compounds were inhibitors of amino acid metabolism in various biological systems.

Phenylalanine Antagonists. Likewise, the structural change (—S— to —CH=CH—) which produced the antithiamin, pyrithiamin, and antagonists of nicotinic acid was applied to phenylalanine. The resultant thienylalanine was found to be an antagonist of phenylalanine for the rat and a number of microorganisms.[35, 36] In each case, phenylalanine reversed the toxicity of thienylalanine. Since thienylalanine can exist as two isomers,

TABLE 1

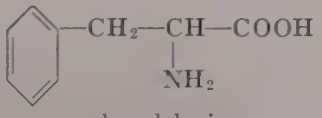
SOME STRUCTURAL CHANGES WHICH HAVE PRODUCED ANTIVITAMINS



2. —S— ⟷ —CH=CH— or —O—, or 

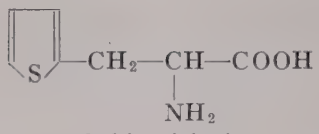
3. CH_3 ⟷ Cl or OH or NH_2 or higher alkyl
4. Pyrimidine ring for benzene ring
5. Interchange of carbohydrates
6. Shift in position of substituents

it seemed desirable to determine whether the β-2- or the β-3-thienylalanine would be the better antagonist. To make this comparison, we synthesized the two isomeric thienylalanines.[72, 40] In addition, the similarity between thiophene and furan compounds suggested that the two isomeric furylalanines should also be prepared and compared with the properties of thienylalanines.

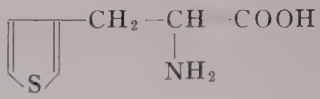


phenylalanine

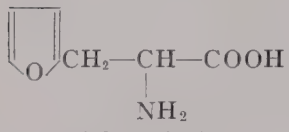
* See footnote (†) TABLE 7, p. 1295.



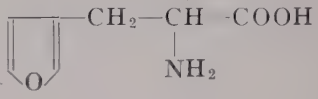
β-2-thienylalanine



β-3-thienylalanine

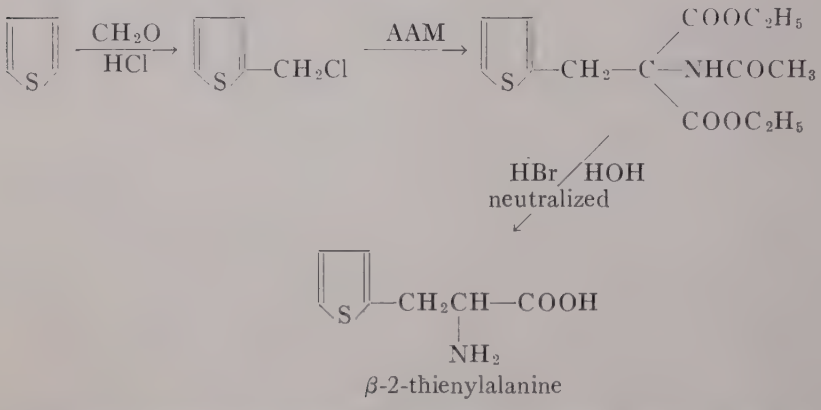


β-2-furylalanine



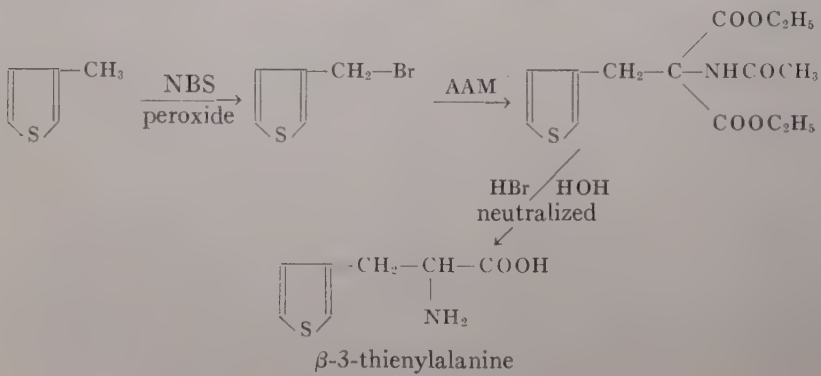
β-3-furylalanine

These four heterocyclic analogues of phenylalanine were synthesized by known methods for the preparation of amino acids. β-2-Thienylalanine was synthesized[72] from 2-chloromethylthiophene and diethyl acetamidomalonate (AAM).[73, 74]



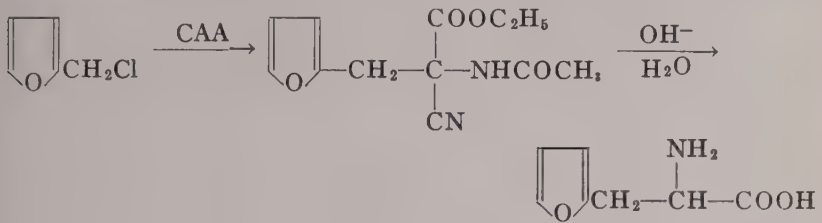
β-2-thienylalanine

β-3-Thienylalanine was prepared[40] in a similar manner, starting with 3-methylthiophene. The side chain was brominated by means of N-bromosuccinimide (NBS), producing 3-bromomethylthiophene, which was reacted with AAM.



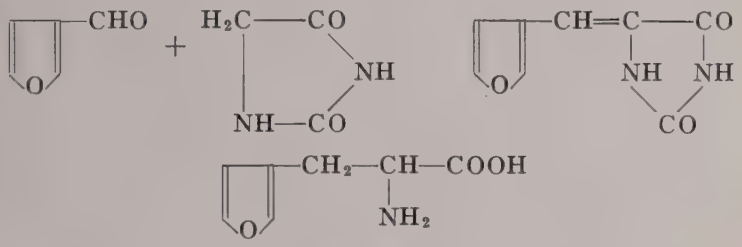
β-3-thienylalanine

β-2-Furylalanine was prepared[75] from furfurylchloride and ethyl cyanoacetamidoacetate (CAA).



β-2-furylalanine

β-3-Furylalanine was prepared[44] by the hydantoin method from 3-furfuraldehyde.



β-3-furylalanine

All four of these heterocyclic analogues of phenylalanine were growth inhibitors for *Escherichia coli* and *Saccharomyces cerevisiae*. The results with β-3-furylalanine are as yet incomplete, but the relative inhibitory powers of the other three are illustrated in FIGURES 1 and 2. It will be noted that, as an inhibitor of the growth of yeast, β-3-thienylalanine is twice as effective as β-2-thienylalanine and seven times more powerful than β-2-furylalanine. Preliminary results indicate that β-3-furylalanine is more potent than β-2-furylalanine. In the presence of $\frac{1}{2}$ optimal levels of phenylalanine, β-3-thienylalanine was again found twice as effective as β-2-thienylalanine as a growth inhibitor of *Streptococcus faecalis R*.

As inhibitors of the growth of *E. coli* (Strain N), β-3-thienylalanine is only slightly more active than β-2-thienylalanine. The activity of β-2-furylalanine is again much less than that of either thiophene analogue (FIGURE 2). The relative amounts of these analogues required to give 50 and 100 per cent inhibition of growth of yeast and *E. coli* are tabulated in TABLE 2.

All the known naturally occurring amino acids were tested for their ability to counteract the toxicity of these heterocyclic antagonists. For each inhibitor and for every microorganism, phenylalanine was most potent in the prevention of the toxicities. Over wide ranges of increasing concentrations of the inhibitors, there was obtained a constant ratio of inhibitor present to phenylalanine required to reverse the inhibition by 50 per cent. (All of the ratios between antagonist and metabolite reported in this paper were obtained at 50 per cent inhibition of normal growth or at the level of

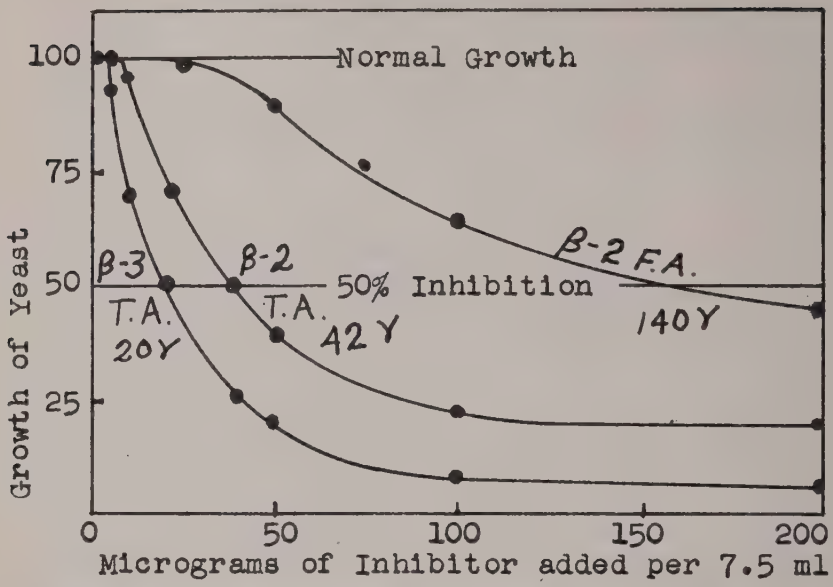


FIGURE 1. The relative inhibitory potencies of β-2-thienylalanine, β-3-thienylalanine, and β-2-furylalanine.

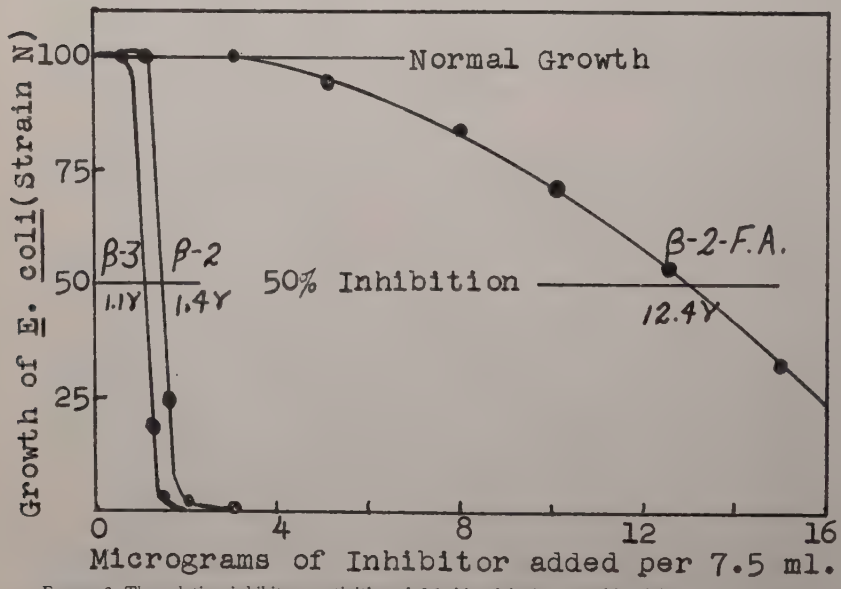


FIGURE 2. The relative inhibitory activities of β-2-thienylalanine, β-3-thienylalanine, and β-2-furylalanine.

growth which represents 50 per cent of the inhibition obtained with inhibitor alone. The amounts of material are expressed as moles.) For yeast, the molar inhibition ratios for β-2-thienylalanine and β-2-furylalanine are 0.83 and 2.0, respectively, and the molar reversal ratios are 0.55 and 1.83.

Tryptophan, leucine, and isoleucine, in addition to phenylalanine, reversed the inhibition of yeast growth by these inhibitors. For *E. coli*, tyrosine and tryptophan were active, while isoleucine and leucine were only very slightly active. For yeast, tyrosine was completely inactive. These differences suggest different metabolic functions of phenylalanine in these two microorganisms.

The comparative studies with these antagonists of phenylalanine demonstrate that, for a metabolite containing a benzene ring, an antagonist might

Table 2

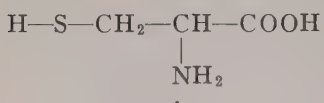
Data Illustrating the Relative Effectiveness of Three Heterocyclic Antagonists of Amino Acids as Microbial Growth Inhibitors

	Amounts of antagonist required per 7.5 ml. medium		
	β-3-thienylalanine	*β-2-thienylalanine*	*β-2-furylalanine*
	γ	γ	γ
S. cerevisiae strain 139			
for 50% inhibition	20	42	140
for 100% inhibition	75	150	>1 mg.
E. coli, strain N			
for 50% inhibition	1.1	1.4	12.4
for 100% inhibition	2	3	20

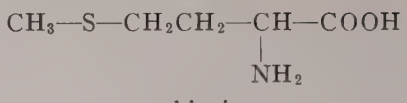
be prepared by replacing the benzene ring with thiophene, putting any required substituent in the 3-position.

Unsaturated Amino Acids as Antagonists. The structural change which resulted in the antagonists, thienylalanine (antiphenylalanine), pyrithiamin (antithiamin), and thiazole carboxylic acid (antinicotinic acid), involved the interchange of an aromatic sulfur (—S—) for a vinylene group (—CH=CH—). This change, with its resultant formation of antagonists of amino acids and vitamins, suggested the desirability of determining whether the replacement of an aliphatic sulfur (—S—) by a vinylene group (—CH=CH—) would result in antagonists of aliphatic sulfur-containing metabolites. Cysteine and methionine, the sulfur-containing amino acids after which the corresponding vinylene compounds were to be molded, were selected.

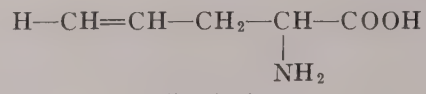
The methallylglycine and crotylglycine were prepared to determine the specificity of the inhibitory properties of allylglycine. The unsaturated amino acids were prepared[51, 52, 76] and tested for their growth-inhibitory properties on three strains of *E. coli* and *S. cerevisiae*, strain 139. The



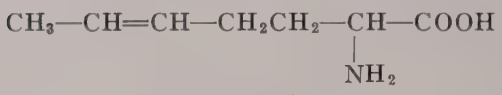
cysteine



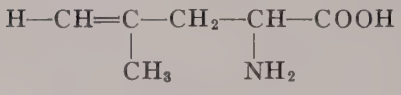
methionine



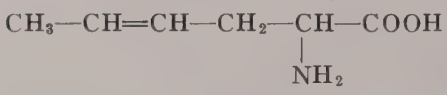
allylglycine



crotylalanine
(2-amino-5-heptenoic acid)



methallylglycine



crotylglycine

effects of allylglycine, methallylglycine, and crotylglycine on the growth of *S. cerevisiae* are plotted in FIGURE 3; and the inhibition of the growth of three strains of *E. coli* by these unsaturated amino acids is represented in FIGURES 4, 5 and 6. From the curves of these figures and the data of TABLE 3, it can be seen that allylglycine and methallylglycine have very similar inhibitory activity, but that crotylglycine is less active for all three strains of *E. coli*. Of the three unsaturated amino acids, allylglycine is by far the best yeast growth inhibitor and crotylglycine is the poorest.

As soon as it was known that allylglycine inhibited the growth of *E. coli*, the effect of cysteine on this growth inhibition was determined. Increasing amounts of cysteine did prevent the toxicity of allylglycine, but so did a number of other amino acids and vitamins at much lower levels than were required of cysteine. In TABLE 4 are listed the various metabolites which have been found to nullify the inhibition of the growth of *E. coli* (ATCC 9723) due to these unsaturated amino acids.

None of the naturally occurring amino acids, when tried singly, reversed the yeast growth inhibition due to allylglycine. The yeast growth inhibition due to methallylglycine was prevented by leucine and valine.

Since the reversal studies with allylglycine and methallylglycine indicated that these two substances were quite different antagonists, further studies on the reversals of their toxicity will be discussed separately.

Reversal of Methallylglycine (MAG) Toxicity. The effect of leucine,

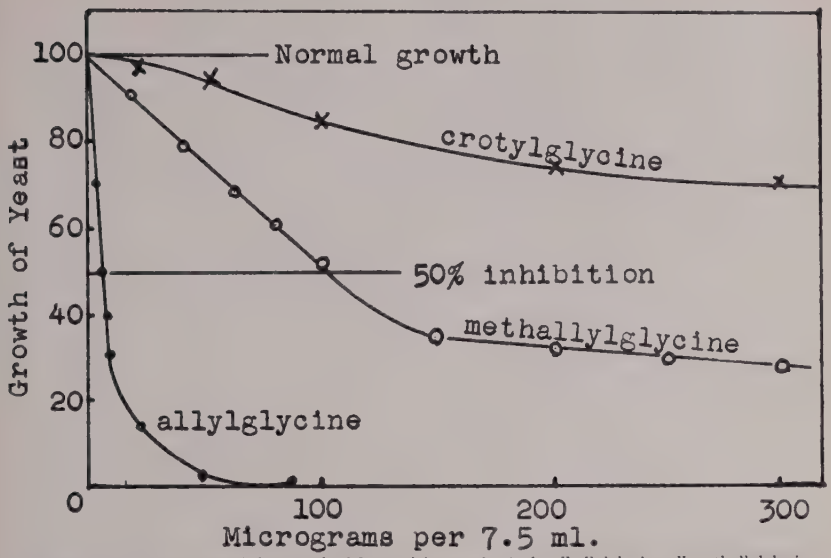


FIGURE 3. The inhibition of the growth of *S. cerevisiae*, strain 139, by *dl*-allylglycine, *dl*-methallylglycine, and *dl*-crotylglycine.

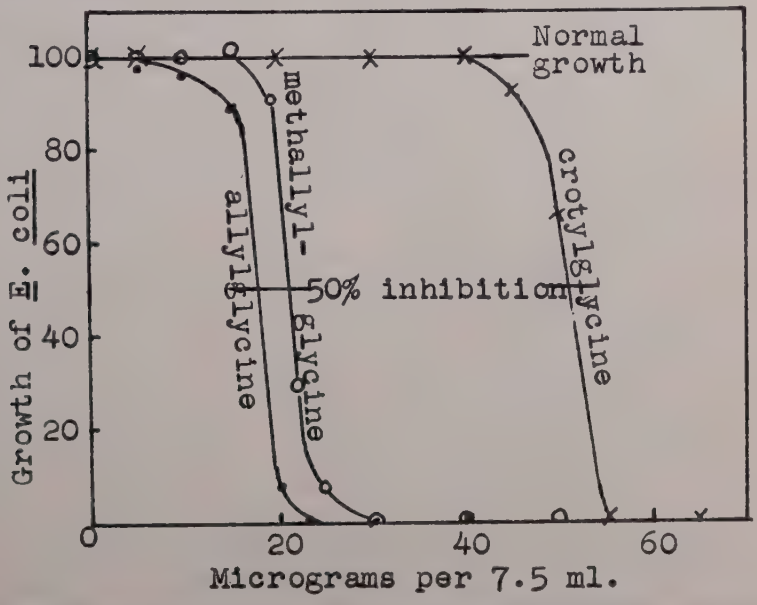


FIGURE 4. The inhibition of the growth of *E. coli*, unidentified strain N, by *dl*-allylglycine, *dl*-methallylglycine, and *dl*-crotylglycine.

valine, tryptophan, and phenylalanine on the inhibition of the growth of *E. coli* (ATCC 9723) by increasing amounts of MAG was determined. It was found that leucine and valine were able to reverse the toxicity over a

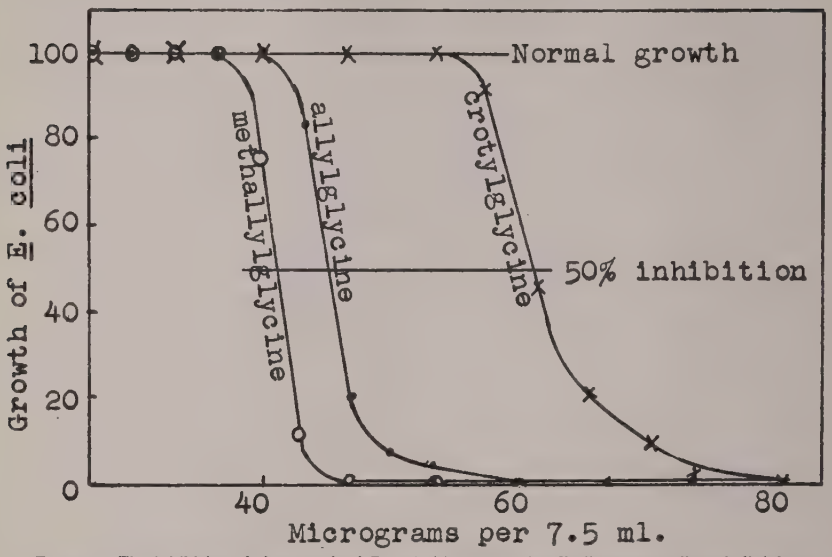


FIGURE 5. The inhibition of the growth of *E. coli*, ATCC 9723, by *dl*-allylglycine, *dl*-methallylglycine and *dl*-crotylglycine.

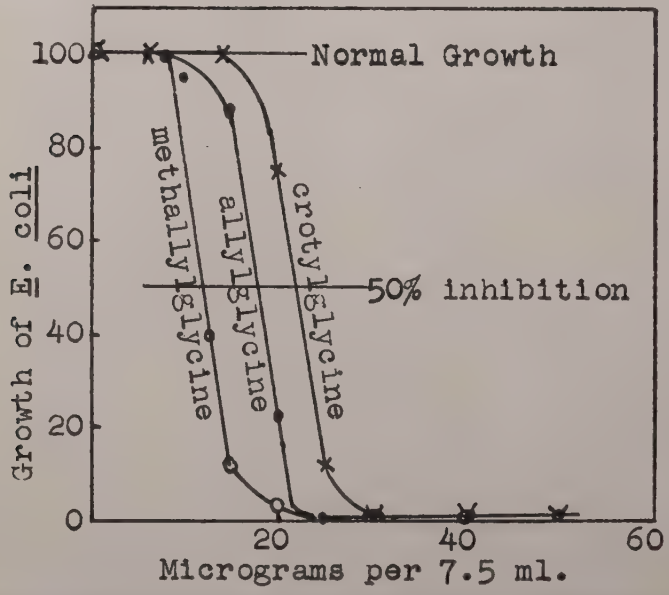


FIGURE 6. The inhibition of the growth of *E. coli*, unidentified strain T, by *dl*-allylglycine, *dl*-methallylglycine, and *dl*-crotylglycine.

large range of MAG. Concentration and a constant ratio of inhibitor to metabolite were maintained. Tryptophan and phenylalanine became less and less effective as the concentration of MAG increased. A constant ratio

TABLE 3

DATA ILLUSTRATING THE RELATIVE EFFECTIVENESS OF THREE UNSATURATED AMINO ACIDS AS MICROBIAL GROWTH INHIBITORS

Microorganism	*Amounts of unsaturated amino acid required* per 7.5 ml. of medium*		
	dl-allylglycine	*dl-methallyl-glycine*	*dl-crotylglycine*
	γ	γ	γ
E. coli, strain N			
for 50% inhibition*	16†	22	50
for 100% inhibition*	20–40	25–40	50–80
E. coli, strain 9723			
for 50% inhibition	27	20	50
for 100% inhibition	30–50	25–40	50–70
E. coli, strain T			
for 50% inhibition	17	10	23
for 100% inhibition	20–30	15–25	30–40
S. cerevisiae, strain 139			
for 50% inhibition	6	55–100	700–1000
for 100% inhibition	50	>1 mg.	>4 mg.

* The amounts required for complete inhibition vary much more than the amounts required for 50 per cent inhibition. The values for 50 per cent inhibition are averages, while the values for 100 per cent inhibition are the range of amounts required.

† For a short period of time during these tests, between 30 and 50 γ were required for 50 per cent inhibition of normal growth.

TABLE 4

AMINO ACIDS AND VITAMINS WHICH NULLIFY THE INHIBITION OF THE GROWTH OF *E. coli* (ATCC 9723) DUE TO UNSATURATED AMINO ACIDS

*Allylglycine (50 γ)**		*Methallylglycine (100 γ)**		*Crotylglycine (100 γ)**
	γ†		γ†	
Methionine	(0.2)	Leucine	(1.3)	Methionine
Tyrosine	(0.8)	Valine	(95)	Tyrosine
Phenylalanine	(4.4)	Tryptophan	(2.6)	Phenylalanine
Leucine	(8.1)	Phenylalanine	(7.6)	Leucine
Valine	(9.0)			Valine
Isoleucine	(12)			Isoleucine
Cysteine	(320)			Cysteine
Glutamic acid	(7300)			Glutamic acid
Thiamin	(0.001)			Tryptophan (?)
Pantothenic acid	(0.005)			Thiamin
				Pantothenic acid

* Micrograms of inhibitor per 7.5 ml. of medium.

† Micrograms of metabolite required to reverse the inhibition to 50 per cent of that obtained with the antagonist alone.

of inhibitor to tryptophan and phenylalanine did not obtain. In TABLE 5 are given the reversal ratios between MAG and three of the metabolites which reverse the growth inhibition of *E. coli* (ATCC 9723).

The inhibition of yeast growth by MAG could be reversed only by leucine,

and, over a large concentration range of MAG, the reversal ratio of MAG over leucine remained constant (see TABLE 6).

The observation that leucine and valine are the only amino acids which prevent the toxicity of MAG and the fact that constant ratios of MAG to leucine and to valine obtain suggest that methallylglycine is an antagonist

TABLE 5
REVERSAL OF METHALLYLGLYCINE (MAG) INHIBITION OF *E. coli*

MAG	*MAG**	*MAG**	*MAG**
γ/tube	*Leucine*	*Valine*	*Tryptophan*
100	43	0.59	23
150	39	0.59	1.87
200	31	0.61	0.28
250	35	0.55	0.21
300	39	0.60	0.09

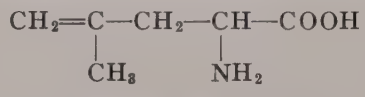
* Molar Reversal Ratio = (a) moles of inhibitor/(b) moles of metabolite: a = amount present minus the amount required to inhibit growth by 50 per cent without added metabolite; b = amount required to reverse the inhibition by 50 per cent.

TABLE 6
REVERSAL OF METHALLYLGLYCINE INHIBITION OF YEAST GROWTH

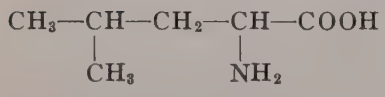
MAG/tube	*MAG**
γ	*Leucine*
500	0.85
800	0.90
1000	0.85
1500	0.92
2000	0.97
5000	1.02

* Molar Reversal Ratio: moles of inhibitor added/moles of metabolite for 50 per cent reversal.

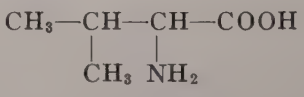
of leucine and, in some organisms, of the structurally related valine. The similarities in structure are evident from the following formulae:



methallylglycine (MAG)



leucine



valine

From these results it would seem that another method of producing metabolite antagonists might be the preparation of the corresponding unsaturated analogue. It is reasonable to expect MAG to be an antagonist of valine also, since the difference is unsaturation and decreasing the carbon chain by one. This represents a double structural change.

Reversal of Allylglycine (AG) Toxicity. In TABLE 4 are listed the amino acids and vitamins which reversed the growth inhibition of *E. coli* (ATCC 9723) by allylglycine. Although cysteine, the metabolite after which the allylglycine was modeled, was effective in preventing the toxicity of AG and the ratio of inhibitor to metabolite remained constant, it must be observed that methionine is much more effective than cysteine in reversing this toxicity. Methionine was effective for all strains of *E. coli*, whereas cysteine was not. The relationship of methionine, thiamin, and pantothenic acid to the effect of allylglycine is under investigation now.

None of the amino acids, when tested singly, prevented the yeast growth inhibition due to AG. A solution of hydrolyzed casein and a mixture of all

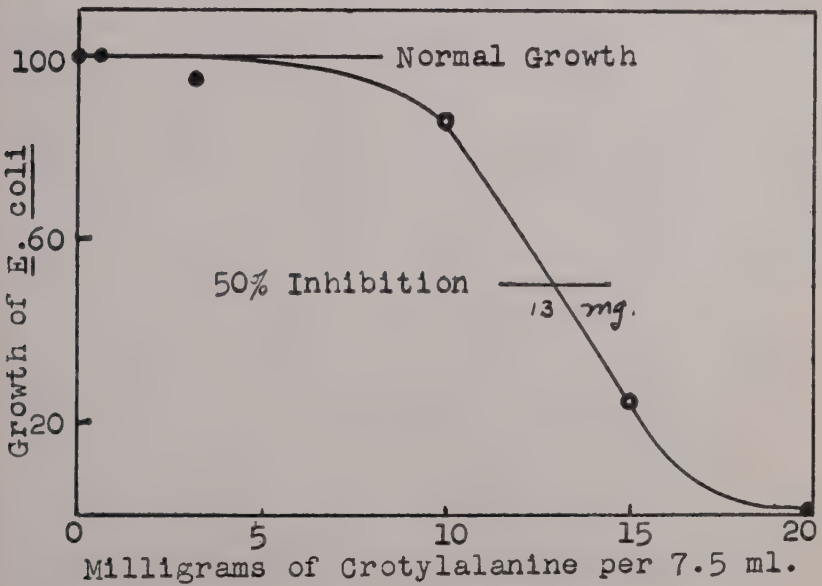


FIGURE 7. The inhibition of the growth of *E. coli*, unidentified strain T, by crotylglycine.

known amino acids prevented the toxicity. A mixture of cysteine, methionine, glutamic acid, phenylalanine, and tyrosine also permitted normal growth in the presence of inhibitory amounts of AG. This is being studied further.

Microbiological Properties of Crotylalanine (CA) (the Vinylene Analogue of Methionine). Increasing amounts of CA inhibited completely the growth of one strain of *E. coli* (unidentified strain T) but did not retard the growth of two other strains of *E. coli* (unidentified N and ATCC 9723) or the growth of *S. cerevisiae*. The effect of CA on the growth of *E. coli* and the complete reversal of this inhibition by methionine are plotted in FIGURES 7 and 8. It will be noted that 13 mg. inhibited growth to 50 per cent of normal and 20 mg. per 7.5 ml. gave 100 per cent inhibition. The toxic effects of 20 mg. CA were completely nullified by 2 micrograms of methionine. At best,

crotylalanine is a very weak methionine antagonist, but, when it did inhibit, it seemed to be specific for methionine.

Analogues of Amino Acids Formed by Replacing a Methyl Group by Chlorine. In 1943, Kuhn[77] reported that 6,7-dichlororiboflavin was a good antiriboflavin, and Woolley[78] found that 2,3-dichloronaphthoquinone was an antagonist of vitamin K. In each of these cases, antagonists resulted when a

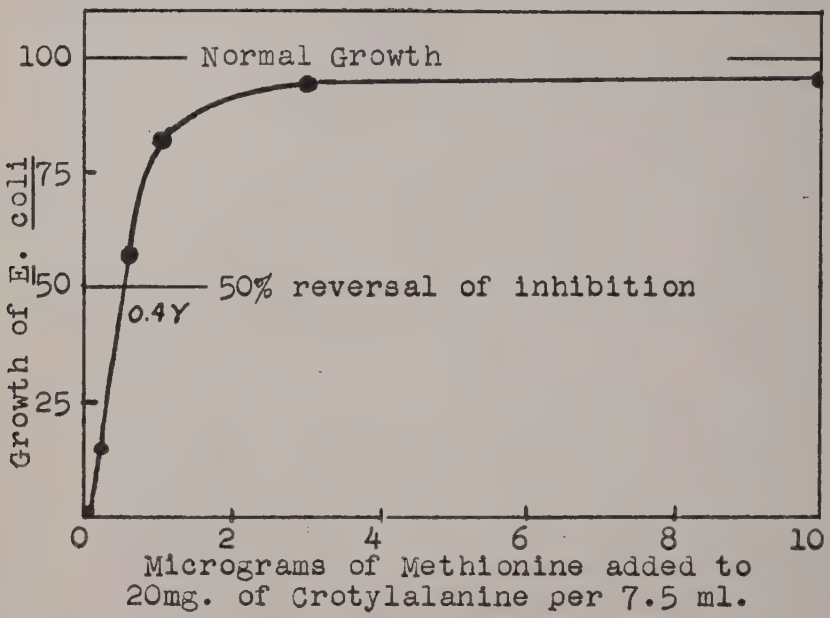
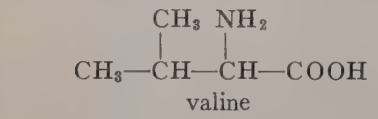
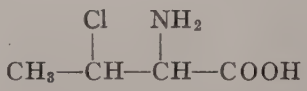


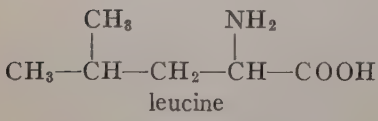
FIGURE 8. Reversal of the toxicity of crotylglycine by methionine.

methyl or other alkyl group was replaced by a chlorine atom. It seemed desirable to determine whether a similar change in some of the amino acids would result in amino acid antagonists. Valine, leucine, and isoleucine were chosen for this structural modification.

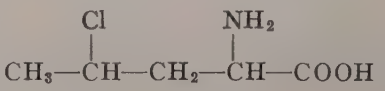


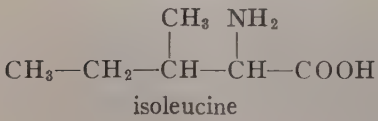
valine



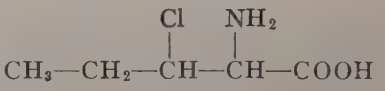


leucine

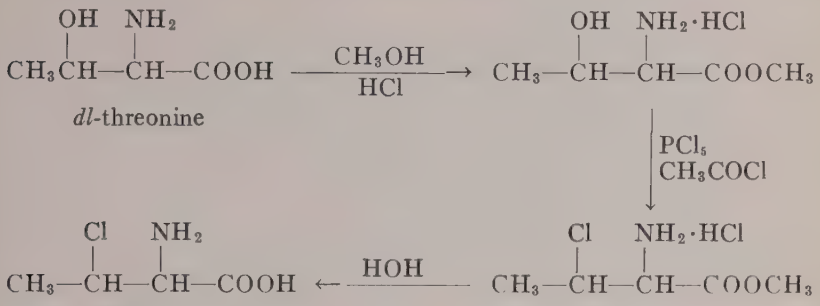




isoleucine



The chloro-analogue of valine has been prepared.[21] It was synthesized from threonine.



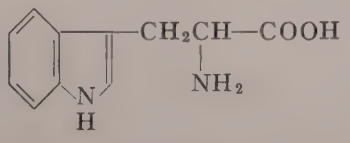
dl-threonine

α-amino-β-chlorobutyric acid

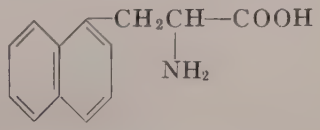
Since the configuration of the carbon atom containing the chlorine might vary, the α-amino-β-chlorobutyric acid was also prepared from *dl*-allothreonine. Both chloro- derivatives were antagonists for the growth of *E. coli* and yeast[21] (FIGURES 9 and 10). The growth inhibition was effectively reversed by the addition of valine, isoleucine, and leucine (FIGURE 11). Several other amino acids also nullified the inhibition but were much less active.

After studying the inhibitory properties and determining which amino acids reverse the toxicity, it is possible to see that α-amino-β-chlorobutyric acid is competing for all three amino acids, valine, leucine, and isoleucine. The change from valine to the chloro- analogue entails the replacement of a CH_3-group by chlorine, whereas the change from leucine or isoleucine to the chloro- analogue includes this same change, in addition to shortening the carbon chain by one methylene group.

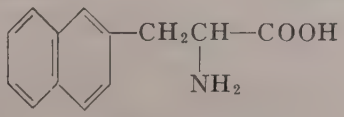
Naphthylalanines. When the benzene ring of phenylalanine was replaced by the pyrrole ring, the resultant pyrrolealanine, although not yet obtained in the pure form, was an antagonist of phenylalanine.[45] Because of this, it seemed important to prepare two isomeric naphthylalanines related to tryptophan by an interchange of the pyrrole portion of indole by a benzene.



tryptophan



β-1-naphthylalanine



β-2-naphthylalanine

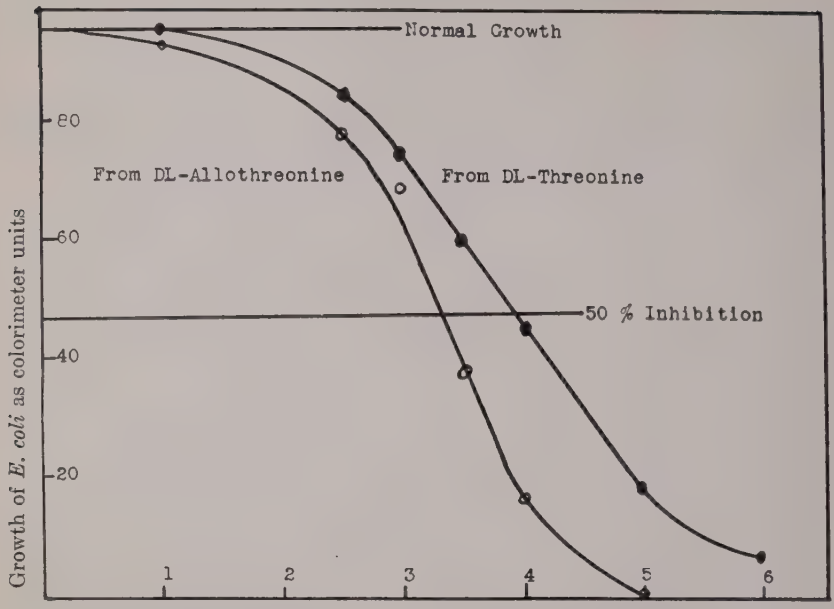


FIGURE 9. Inhibition of *E. coli* by two isomeric β-chloro-α-aminobutyric acids.

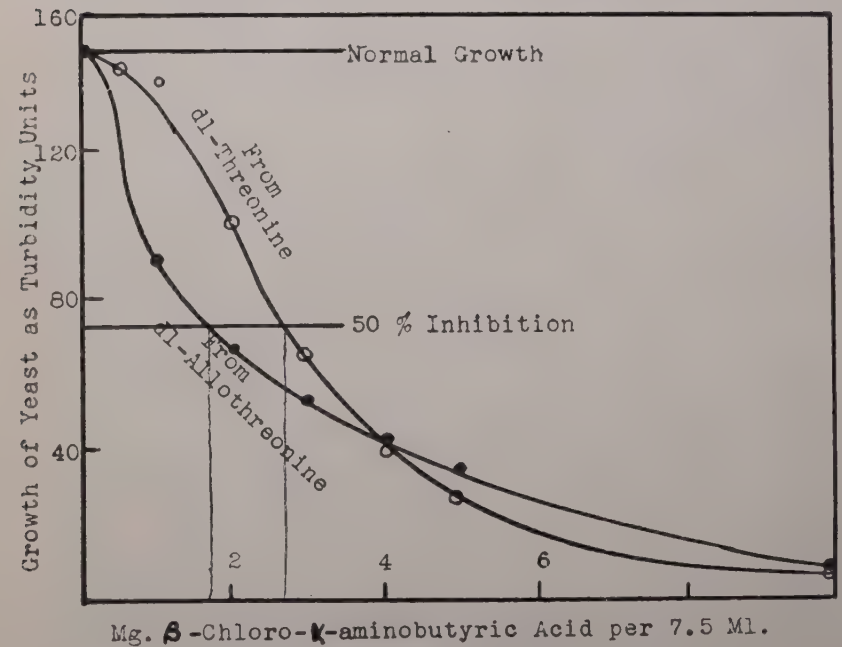


FIGURE 10. Inhibition of the growth of yeast by two isomeric β-chloro-α-aminobutyric acids.

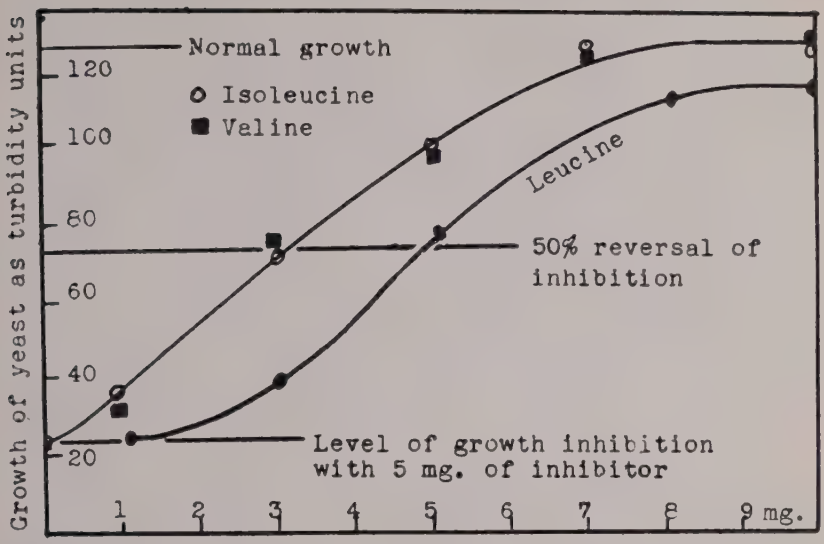


FIGURE 11. Effect of valine, leucine, and isoleucine on toxicity of 5 mg. of β-chloro-α-aminobutyric acid from *dl*-threonine.

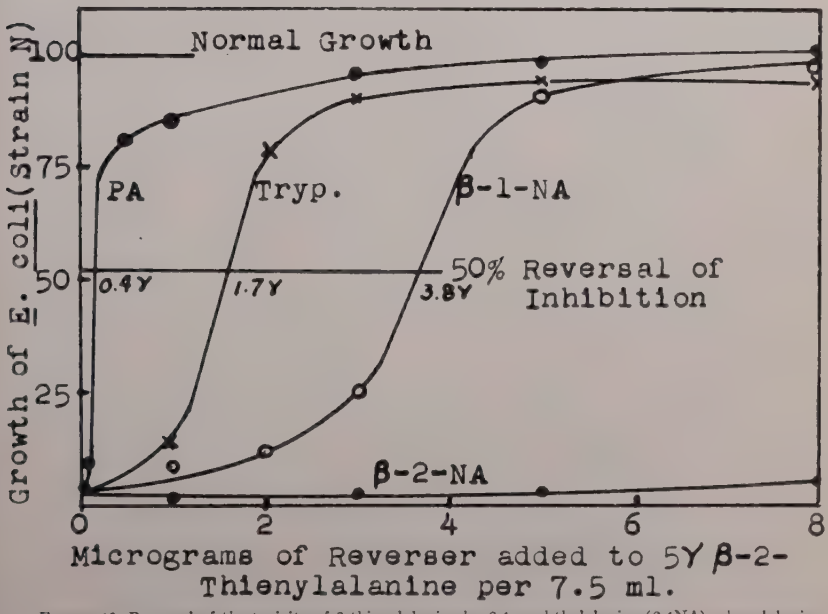


FIGURE 12. Reversal of the toxicity of β-thienylalanine by β-1-naphthylalanine (β-1NA), phenylalanine (PA), and tryptophan (tryp).

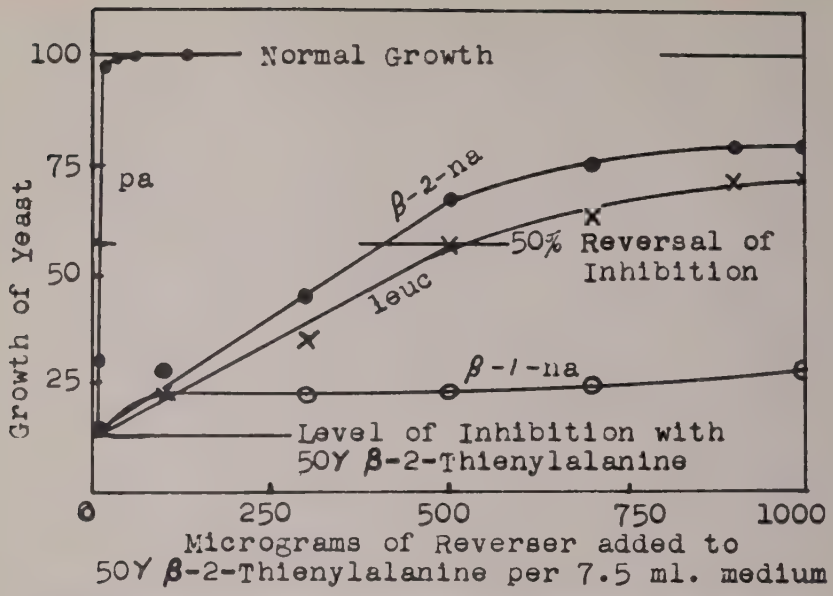


FIGURE 13. Reversal of the toxicity of β-2-thienylalanine by β-2-naphthylalanine (na), leucine (leuc), and phenylalanine (pa).

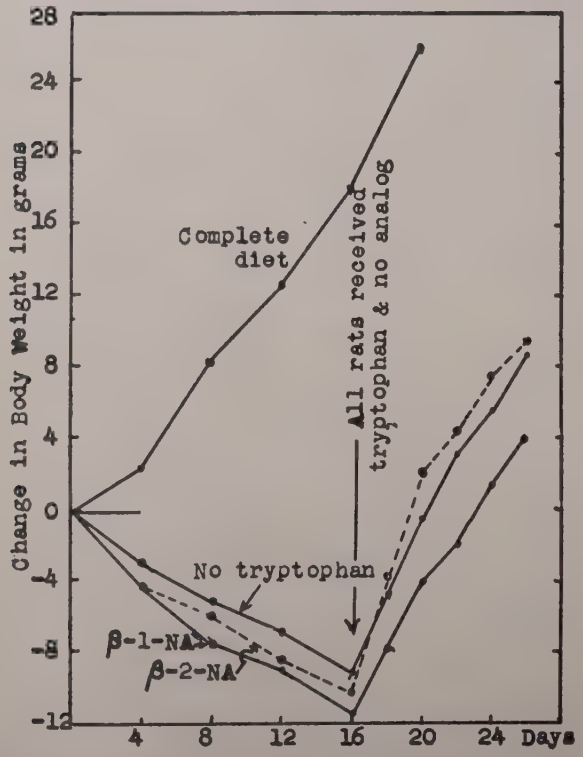


FIGURE 14. Growth curves of rats fed β-1- and β-2-naphthylalanines in place of tryptophan. Each group contained 5 rats.

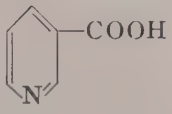
The two isomeric naphthylalanines were tested for their ability to inhibit the growth of microorganisms, and both were inactive at concentrations up to 5 mg. per 7.5 ml. medium. Neither would they substitute for tryptophan in organisms which required tryptophan for normal growth. When these two analogues were tested for their ability to reverse the toxicity of thienylalanine, however, it was found that β-1-naphthylalanine reversed the inhibition of the growth of *E. coli* and β-2-Naphthylalanine was inactive (FIGURE 12). The inhibition of the growth of yeast due to thienylalanine was almost completely nullified by β-2-naphthylalanine, while β-1-naphthylalanine was almost completely inactive (FIGURE 13). To prevent the toxicity of thienylalanine on the growth of yeast phenylalanine was best and leucine was the next most active reverser. β-2-Naphthylalanine is more active than leucine if compared on a molar basis.

These results suggest some role of the naphthylalanines in amino acid metabolism. To obtain further information about what that role might be, these analogues were fed to rats on a tryptophan-deficient diet.[50] No significant variations in the weight changes were observed (FIGURE 14).

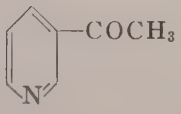
Phenyl-Ketone Analogues of Amino Acids. Auhagen,[79] in 1942, found that *p*-aminoacetophenone was an antagonist of PAB. This structural change also produced antagonists of nicotinic acid[80] and of pantothenic acid.[81] Woolley found that the methyl ketone analogue of pantothenic acid had little inhibitory power, whereas the phenyl ketone was a potent antagonist.

PAB

PAB-antagonist

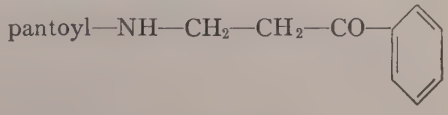


nicotinic acid



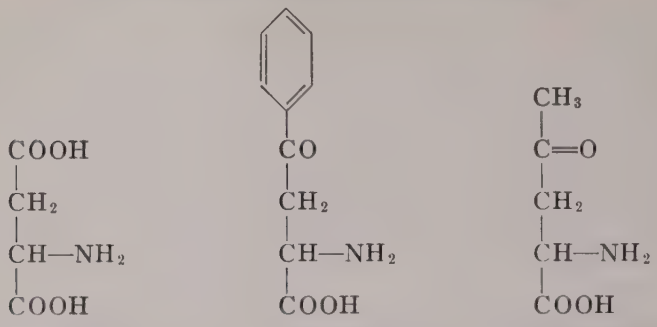
nicotinic acid antagonist

pantothenic acid



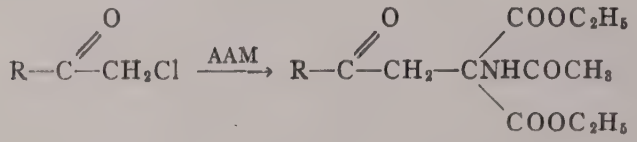
phenyl pantothenone (antagonist)

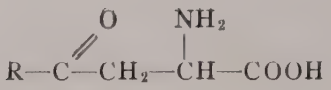
The dicarboxylic amino acids seemed suitable structures to convert to the corresponding methyl and phenyl ketones to ascertain whether that change would result in antagonism of these amino acids.

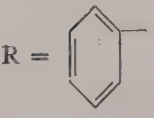


aspartic acid aspartophenone α-aminolevulinic acid

Aspartophenone and α-aminolevulinic acid were synthesized[58] according to the following reactions:







for aspartophenone

R = —CH_3 for α-aminolevulinic acid

After a few experimental trials with these ketone analogues of aspartic acid, it was observed that both were inhibitory but that the potency of aspartophenone was dependent on heating the molecule. In fact, without some heat treatment the compound is completely inactive for *E. coli* and has only slight activity on the growth of yeast. The effect of heat on the activity as a growth inhibitor of *E. coli* and yeast is illustrated in FIGURES 15 and 16. The activity of α-aminolevulinic acid was not affected by heating. The effect of heat suggests that the crystalline compound exists in some inactive form.

When maximum activity is obtained following the heat treatment, the aspartophenone is a potent inhibitor of the growth of *E. coli* and yeast. It is much more active than the corresponding methyl ketone analogue, α-aminolevulinic acid (FIGURES 17 and 18).

Enzyme Studies. Some of the antagonists described have been tested for their ability to inhibit isolated enzyme systems. Thienylalanine, furylalanine, and allylglycine have been tested for their effect on *E. coli* lactic dehydrogenase activity. When the crude enzyme was prepared by most common procedures, inhibition of O_2 uptake was observed due to added

antagonists, which could be reversed by the simultaneous addition of the corresponding metabolite. This inhibition, however, was correlated with

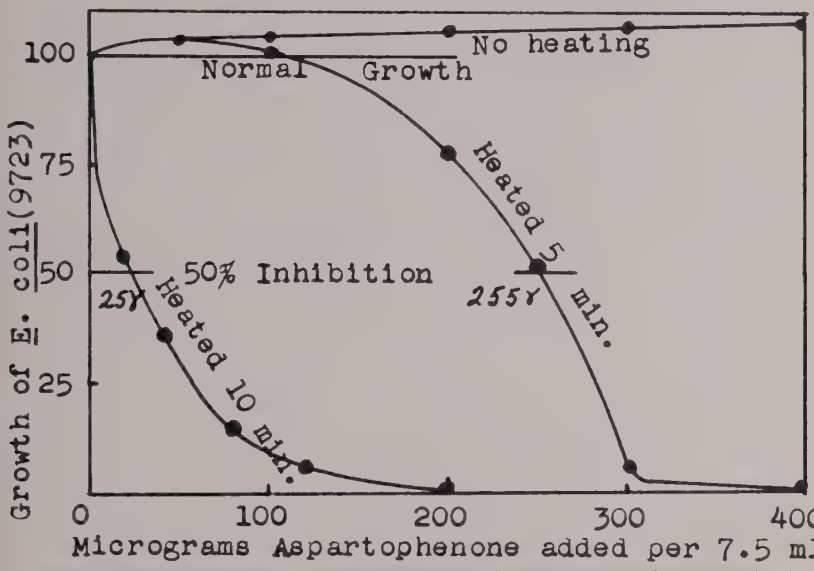


FIGURE 15. The effect of heating on the inhibitory activity of aspartophenone on the growth of *E. coli*.

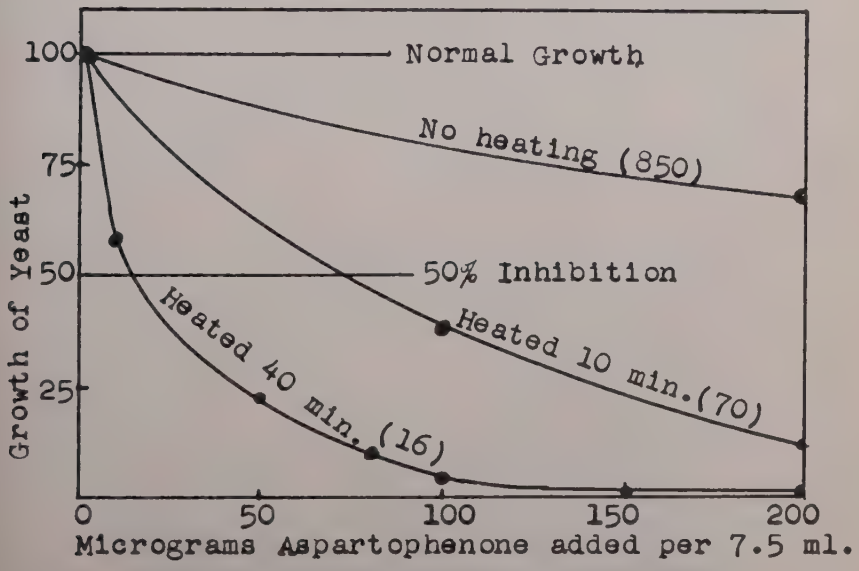


FIGURE 16. The effect of heating on the inhibitory activity of aspartophenone.

inhibition of cell multiplication and growth. Finally, Saburo Katsura, of our laboratory, developed a simple method for preparing a cell-free en-

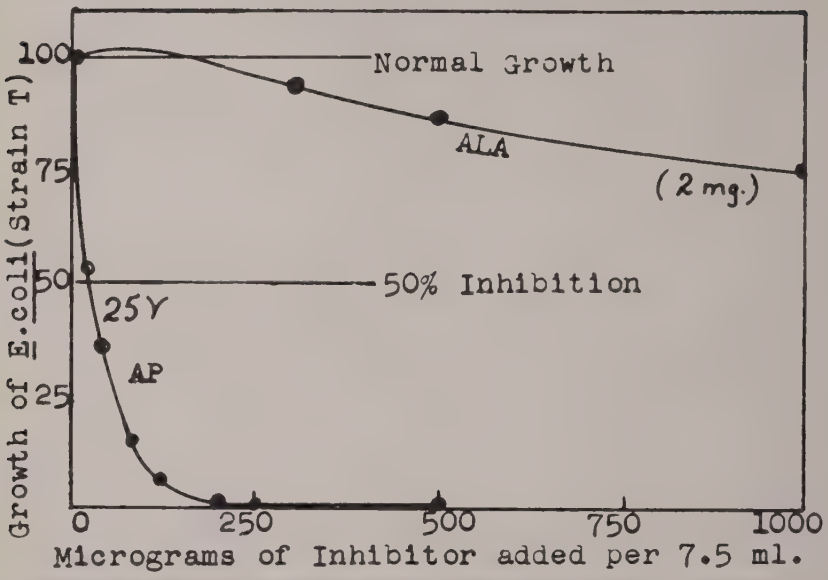


FIGURE 17. A comparison of inhibitory properties of aspartophenone (AP) and α-aminolevulinic acid (ALA).

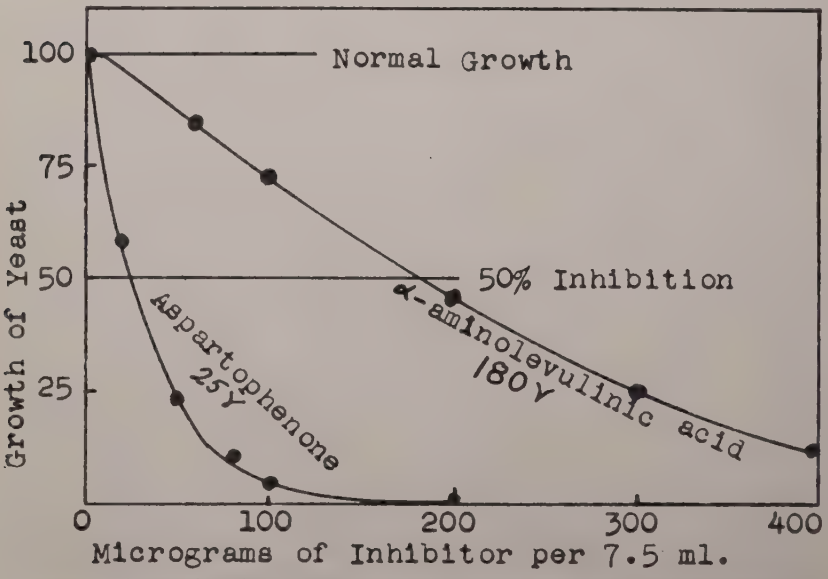


FIGURE 18. A comparison of the inhibitory properties of aspartophenone and α-aminolevulinic acid.

zyme preparation. Such a preparation was easily prepared by shaking a suspension of *E. coli* cells with chloroform. The remaining material contained the enzyme activity and no viable cells. With this enzyme prepara-

TABLE 7
AMINO ACID ANTAGONISTS*

Structural change and amino acid altered	*Analogue*	*Inhibition*	*Biological system*	*Reference*
I. —COOH to —SO_3H				
glycine	α-aminomethane sulfonic acid	+	bacteriophage	5
		+	vaccinia virus	6
		+	bacteria	7
		−	*E. coli*	8†
alanine	α-aminoethane sulfonic acid	+	bacteria	7
		−	*E. coli*	8†
		−	mouse tumor	9
valine	α-aminoisobutane sulfonic acid	+	bacteria	7, 9, 10
		+	vaccinia virus	6
leucine	α-aminoisoamyl sulfonic acid	+	bacteria	7, 10
		−	mouse tumor	9
aspartic acid	cysteic acid	−	bacteria	7, 10
		+	bacteria	11
phenylalanine	α-amino-β-phenylethane sulfonic acid	−	mouse tumor	9
II. —CH_3 to —H				
alanine	glycine	+	bacteria	12
valine	α-aminobutyric acid	+	bacteria	8, 13
leucine	norvaline	+	bacteria	8‡
threonine	serine	+	bacteria	13, 14
III. —H to —CH_3				
methionine	ethionine	+	rats	15
		+	bacteria	16
serine	α-methylserine	−	bacteria	8†
tryptophan	methyltryptophans	+	bacteria	17, 18
			bacteriophage	19
alanine	α-aminoisobutyric acid	−	bacteria	8
IV. —CH_3 Shifted in position				
valine	norvaline	+	bacteria	8‡
leucine	norleucine	+	bacteria	7, 8‡, 70
isoleucine	leucine	+	bacteria	20

* A list of various structural changes and the amino acids which have been altered accordingly to form amino acid antagonists. The biological systems tested for the possible inhibitory activity are listed; inhibition is indicated by a (+), while lack of inhibition is indicated by a (−).

† The compounds used in these experiments were kindly supplied by Dr. Marvin D. Armstrong, School of Medicine, University of Utah, Salt Lake City, Utah.

‡ The compounds used in these experiments were kindly supplied by Dr. Julius E. Johnson, Biochemical Research Laboratory, Dow Chemical Company, Midland, Michigan.

TABLE 7—(*Continued*)

Structural change and amino acid altered	*Analogue*	*Inhibition*	*Biological system*	*Reference*
V. —CH_3 to —Cl				
valine	α-amino-β-chloro-butyric acid	+	bacteria, yeast	21
VI. Increase C-chain by one —CH_2—				
valine	leucine isoleucine	+	bacteria	13, 14, 22, 71
serine	homoserine	+	bacteria	8‡
tyrosine	α-amino-γ-(*p*-hydroxy-phenyl)-butyric acid		not tested	23
VIIA. —H to —OH				
alanine	serine	+	bacteria	12
aspartic acid	hydroxyaspartic acid	+	bacteria	24
phenylalanine	tyrosine	+	bacteria	25
phenylalanine	β-hydroxyphenylalanine	+	bacteria	26
proline	hydroxyproline	+	fungi	27
VIIB. —H to —NH_2				
aspartic acid	diaminosuccinic acid	+	bacteria	24
VIII. —OH to —NH_2 and —NH_2 to —OH				
glutamic acid	glutamine	+	bacteria	28
tyrosine	*p*-aminophenylanine	+	fungi	29, 30
lysine	α-amino-ε-hydroxy-caproic acid	+	rat	31
ornithine	α-amino-δ-hydroxy-valeric acid	−	bacteria	32
IX. —H to —F and other halogens				
phenylalanine	fluorophenylalanines	+	fungi papain enzyme	33 34
	chlorophenylalanines	+	fungi	33
	bromophenylalanines	+	fungi	33
tyrosine	fluorotyrosines	+	fungi	33
	3,5-difluorotyrosine	+	fungi	33

TABLE 7—(*Continued*)

Structural change and amino acid altered	*Analogue*	*Inhibition*	*Biological system*	*Reference*
X. Aromatic —CH=CH— to —S—, —O—, —NH—, and —N=CH— and *vice versa*.				
phenylalanine	β-2-thienylalanine	+	rat and yeast	35
		+	bacteria and yeast	26, 36, 37
		+	vaccinia virus	38
		+	tubercle bacilli	39
	β-3-thienylalanine	+	bacteria and yeast	40, 41
	β-2-furylalanine	+	bacteria, yeast	42, 43
	β-3-furylalanine	+	bacteria, yeast	44
	β-2-pyrrolealanine	+	bacteria, yeast	45
	β-4-pyridylalanine	+	bacteria	30, 46
tryptophan	naphthylalanines	−	bacteria	47, 48, 49
		−	rat	50
XI. Aliphatic —S— to —CH=CH—				
cysteine	allylglycine	+	bacteria, yeast	51
methionine	2-amino-5-heptenoic acid	+	*E. coli*	52
XII. Aliphatic —S— to —O— and to —CH_2—				
methionine	methoxinine	+	bacteria	53
		+	vaccinia virus	6
	norleucine	+	bacteria	16, 54
XIII. Saturated bonds to unsaturated bonds with other changes				
isoleucine	methallylglycine	+	bacteria, yeast	51, 55
leucine, with shift of —CH_3	methallylglycine	+	bacteria, yeast	51, 55
valine, with C-chain lengthened	methallylglycine	+	bacteria, yeast	51, 55

TABLE 7—(*Continued*)

Structural change and amino acid altered	*Analogue*	*Inhibition*	*Biological system*	*Reference*
Tryptophan, with indoleacrylic acid —NH_2 to —H		+	bacteria	56
Tryptophan, with naphthylacrylic acid —NH_2 to —H and —NH— to —CH=CH—		+	bacteria	57
XIV. —COOH to phenylketones and methylketones:				
aspartic acid	aspartophenone	+	bacteria, yeast	58
	α-aminolevulinic acid	+	bacteria, yeast	58
XV. Optical Inversion				
leucine	D-leucine	+	bacteria	59
histidine	D-histidine	+	histidase	60
XVI. Miscellaneous changes:				
aspartic acid	—COOH to —H, β-alanine	+	yeast	14
glutamic acid	—COOH to —$SOCH_3$, methionine sulfoxide	+	enzyme	69
		+	bacteria	61, 62
	—COOH to —CON-HC_2H_5, N-(γ-glutamyl)-ethylamine	+	bacteria	63
arginine	—CH_2— to —O— canavanine	+	fungi, bacteria	32, 64
	—C(=NH)—NH_2 to —H, lysine	+	arginase enzyme	65
ornithine	—CH_2— to —O—, canaline	−	bacteria	32
lysine	—H to —C(=NH)—NH_2, arginine	+	fungi	66

TABLE 7—(*Continued*)

Structural change and amino acid altered	*Analogue*	*Inhibition*	*Biological system*	*Reference*
phenylalanine	benzene ring to indole ring, tryptophan	+	bacteria	67
tryptophan	—NH— to —S—, β-(2-benzothienyl)-alanine	+	bacteria	68
tryptophan	—NH— to —OC=O—, β-3-coumaronyl-alanine		not tested	48
histidine	alanine side-chain to —H, imidazole		histidase	60
XVII. Double changes in the same molecule:				
valine	methallylglycine (unsaturation and increase in C-chain)	+	yeast, bacteria	55
leucine	methallylglycine (unsaturation and shift in $—CH_3$)	+	yeast, bacteria	55
leucine	α-amino-β-chlorobutyric acid ($—CH_3$ to —Cl and shortened C-chain)	+	yeast, bacteria	21
tryptophan	indoleacrylic acid (See above)	+	bacteria	56
tryptophan	naphthylacrylic acid (see above)	+	bacteria	57

tion none of the inhibitors retarded oxygen uptake of this dehydrogenase enzyme system. Further enzyme studies are underway.

Summary

A number of the structural changes of various vitamin molecules which led to the production of antivitamins have also been applied to amino acids. In many instances, the analogue was inhibitory. Many of the structural changes which have been applied to amino acids are catalogued in TABLE 7. This table indicates the structural modification, to what amino it has been applied, and whether or not that structural change produced antagonism.

It is evident that many of these changes have to be applied to many more compounds before their general applicability can be established. Some have been applied quite extensively, and certain generalizations about effectiveness can be predicted wherever similar changes are made.

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DESOXYPYRIDOXINE OBSERVATIONS IN "ACUTE PYRIDOXINE DEFICIENCY"*

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Pyridoxine deficiency induced by the feeding of a diet deficient in vitamin B_6 was first described in rats by Goldberger and Lillie[1] and György.[2] The changes in this condition as they occur in a variety of species have been reviewed recently.[3] The deficient state in such animals was the result of a gradual depletion in pyridoxine which is referred to here as "chronic pyridoxine deficiency." In contrast, the nutritional disease produced by the administration of a pyridoxine antagonist, due to "blockade" of vitamin B_6, may be considered to result from an "acute pyridoxine deficiency."

2,4-dimethyl-3-hydroxy-5-hydroxy-methyl pyridine, a desoxypyridoxine (D. B_6), was first seen by Ott to exert antivitamin B_6 activity in the chick.[4] In this species, the inhibitory ratio at low levels of pyridoxine intake was 2:1. In rats, Emerson has shown D. B_6 to be effective against pyridoxine and pyridoxal.[5] Interestingly enough, the same author found that one member of the vitamin B_6 group, pyridoxamine, could not be antagonized by D. B_6.[6] On purified diets, devoid of pyridoxine, it has been observed that biochemical[7] and morphological[8] changes characteristic of prolonged pyridoxine deprivation may be produced by D. B_6 almost immediately after its administration.

Previous experiments have shown that vitamin B_6 is more essential than other dietary constituents for the maintenance of lymphoid tissue[8, 9] and for the development of antibody responses.[10] Accordingly, it was found that the administration of D. B_6 caused marked atrophy of lymphoid tissue[8, 11] and regression of lymphosarcoma implants.[12] Also, antibody responses were severely impaired in "acute pyridoxine deficiency."[13] These findings have been extended, and the effects of pyridoxine deprivation produced by the administration of D. B_6 have been further explored in the experiments summarized in the following.

Mice of the C and C3H strain and Sherman rats were used for the experimental groups listed in the tables and figures. The composition of the diets was the same as in previous experiments.[10] For mice, inosital and P.A.B.A. were added to the rat ration. Unless stated differently, D. B_6§ was administered in the drinking water (0.3 mg. per cc.). About 3 cc. of water was consumed daily by mice and up to 20 cc. by rats. Adrenalectomized animals were maintained on saline. Tissues were fixed and bones decalcified in Bouin's solution for histological examination. In 2 groups of rats, the adrenals were prepared for cytochemical studies of steroids by Dr. Helen W. Dean. Fragments of livers of 32 rats and of 21 mice were assayed

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§ Supplied by Merck & Co., Inc.

after extraction in 0.05 N HCl for vitamin B_6 on the neurospora "pyridoxine-less."[14] Pyridoxal phosphate activity was determined on tyrosine decarboxylase from 15 livers of the same rats by Dr. W. W. Umbreit. Antibodies (hemagglutinins) were titrated by the usual method of serum dilution in 2-fold steps.

Growth retardation, dermatitis, hyperirritability, hunching of the back, prostration, and death were observed in young rats (50–80 gm. of body

FIGURE 1. Rat after 3 weeks of "acute pyridoxine deficiency." In addition to the customary changes there is dermatitis and inward curling of the tail.

weight) after prolonged feeding of a diet deficient in vitamin B_6. All of these symptoms occurred much sooner when D. B_6 was administered to comparable animals. Similarly treated older rats (100–300 gm. of body weight) showed severe manifestations of the nutritional disease within 2–3 weeks, while animals of the same age fed the deficient diet alone exhibited but slight evidence of pyridoxine deficiency after several months. All of the symptoms subsided following the administration of pyridoxine-HCl.

In rats with "acute pyridoxine deficiency," a symptom was observed that

was not seen even in the most severe stages of the "chronic deficiency." Rats with "acute pyridoxine deficiency" exhibit dermatitis of the tail with curling in of the distal, ventral aspect of the latter (FIGURE 1). Histologically, the dermatitis of the tail is not different from that seen in the chronic deficiency at the ears, paws, and snout.[14] There also was marked akanthosis, parakeratosis, and hyperkeratosis. In advanced cases, superimposed infection with cellulitis extending into the subcutaneous tissues was not infrequently noted. Usually, such inflammatory changes appeared to have had, as a starting point, the accessory structures of the skin. The inward curling of the tail in this condition is a phenomenon that can not be readily explained by a distinct anatomical substrate. The fact that the contracture persists during deep anesthesia and even after death appears to eliminate the possi-

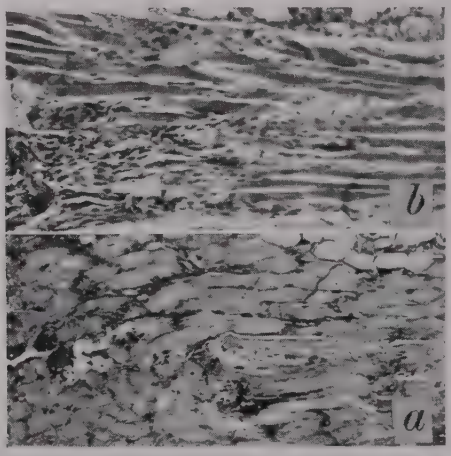

FIGURE 2. (a) Low power view of skeletal muscle of the distal portion of the tail of a control animal. (b) Similar tissue from a rat rendered "acutely" deficient. There is marked fibrosis and atrophy of the muscle fibers with an apparent increase in nuclei.

bility of its being neurogenic in origin. The mechanics of the rat's tail are governed by contractions of muscles with long tendinae and of such muscles that extend directly from one vertebral body of the tail to another. The portion of the tail involved is mobilized predominantly by skeletal muscles without the mediation of tendinae. These muscles present rather striking histologic evidence of atrophy and fibrosis (but not of myositis) (FIGURE 2), and it appears plausible that this myopathy may be the cause for the peculiar posture of the tail. Bending of the vertebral bodies (FIGURE 3) became evident when the lesions existed for some time. The bony changes, however, must be considered as a consequence rather than a cause of the deformity.

In mice, except for the more rapid development of the symptoms, there was no grossly apparent difference in the manifestations of the "acute" and the "chronic" type of pyridoxine deficiency.

The question as to whether the vitamin content of animal tissues is altered

following the administration of a related antivitamin has undoubtedly been raised many times. In most cases, however, this question must remain unanswered, since microbiological assays, as well as chemical determinations, are usually invalidated by the presence of a chemically related antagonist. This situation, however, is different in the case of D. B_6. Here it was seen that, over the entire range of assay, even large amounts of D. B_6 do not alter the growth response of "neurospora pyridoxine-less" to pyridoxine HCl.

Therefore, the vitamin B_6 contents of organs of animals treated with D. B_6 could be determined and were compared with those of controls. The animals treated with D. B_6 showed marked symptoms of pyridoxine deprivation, while the controls were grossly normal. It is seen from TABLE 1 that

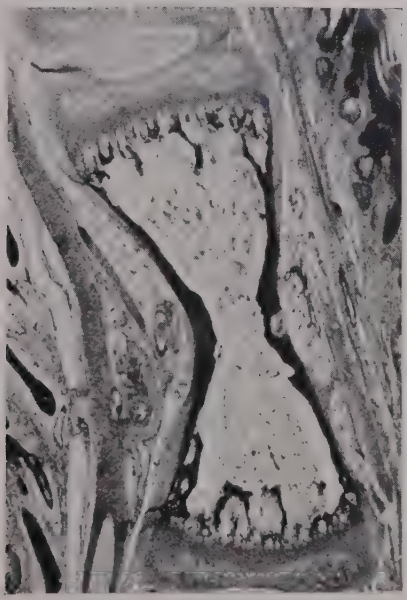

FIGURE 3. Same rat as in FIGURE 2b. A low power view showing curvature of caudal vertebra.

the livers of rats and mice receiving D. B_6 for 2 weeks, while being fed a diet deficient in vitamin B_6, contained as much vitamin B_6 as those of normal animals and more than those of comparable animals not receiving the antagonist. In part of the livers, pyridoxal PO_4 was determined on tyrosine decarboxylase activity by Dr. Umbreit. Pyridoxal phosphate also was relatively increased in the "acute deficiency" and reduced in the "chronic deficiency."

It was recently found by Umbreit that tyrosine decarboxylase is not inhibited by D. B_6 unless the latter is converted to D. B_6-phosphate, which competes with pyridoxal phosphate for the free enzyme surface. It has been suggested by the author that, likewise in animals, the phosphorylated analogue may be the effective moiety.[15] The relative increase in liver pyri-

doxal phosphate found in animals with "acute pyridoxine deficiency" appears compatible with this conception.

Previously, it was demonstrated in mice that the feeding of D. B_6 in combination with a diet devoid of vitamin B_6 produces distinct lymphoid atrophy within a few days.[8] In recent studies by Mushett and co-workers, this has been confirmed in a variety of species.[11] It remained, however, to be shown that this effect of D. B_6 was related to its antivitamin activity and not to a nonspecific toxic action. It also appeared of interest to investigate whether the effect of pyridoxine deficiency upon lymphoid atrophy was

TABLE 1
DESOXYPYRIDOXINE ON VITAMIN B_6 AND ON PYRIDOXAL PO_4 CONTENT OF LIVER*

Group	B_6	DB_6	*B_6 (neurospora) γ/gm.*		*B_6-al PO_4 (tyros. decarb.) γ/gm.*		*No. of animals*
			Av.	*Range*	*Av.*	*Range*	
A	+	−	9.7	8.3–12.2	—	—	5 Mice
B	−	−	8.4	7.3–10.7	—	—	8 "
C	−	+	11.8	9.6–13.2	—	—	8 "
D	+	−	13.1	8.4–14.8	3.6	1.7–4.1	11 Rats
E	−	−	8.4	6.0–12.6	1.6	0.7–2.8	11 "
F	−	+	11.7	7.3–15.7	2.4	1.1–3.4	10 "

* All animals 2 weeks on experiment.

TABLE 2
DESOXYPYRIDOXINE ON BODY WEIGHT AND THYMUS WEIGHT*

Group	*mg. of*		*gm.*		*gm. thymus wt.*	*gm. spleen wt.*	*No. of mice*
	B_6	*Desoxy B_6*	*I.B.W.*	*F.B.W.*			
A	—	—	24.0	25.0	0.033	0.081	5
B	—	.03	23.4	18.2	0.014	0.053	5
C	—	.15	24.2	18.5	0.014	0.052	5
D	—	.30	24.2	17.5	0.011	0.046	5
E	—	1.50	24.4	20.1	0.013	0.050	5
F	.30	.90	24.3	25.8	0.032	0.078	5
G	†	—	25.0	26.0	0.030	—	5

* All animals 1 week on experiment.
† Stock Diet.

mediated by adrenal cortical hyperactivity. The latter possibility had to be considered because of the widely held belief that changes in the quantity of lymphoid tissue occur mainly under the influence of pituitary and adrenal cortical secretion.

The data summarized in TABLE 2 show that, in mice fed a diet deficient in pyridoxine, the daily administration of 0.03 mg. of D. B_6 produces loss of body weight and of lymphoid tissue. Both these events fail to occur when the deficient diet is fed alone or when pyridoxine-HCl is given in addition to the antivitamin. Increasing the quantities of the analogue from 5–50 times caused no additional losses of body weight or lymphoid tissue. These facts

indicate that, at the doses employed, it is only the antivitamin activity of D. B_6 which is responsible for the observed effects. A comparison of adrenalectomized and intact rats treated with D. B_6 (TABLE 3) reveals that lymphoid atrophy in pryidoxine deficiency occurs without intermediation of the adrenal cortex. Also, no changes in the weight or in the morphology of the adrenal gland were observed following the administration of desoxy B_6. In rats fed a diet deficient in pyridoxine, the administration of D. B_6 over 3 weeks (TABLE 4) produced moderate weight losses, extreme thymic atrophy, and severe dermatitis. When the antagonist was withdrawn 10

TABLE 3

THYMUS ATROPHY IN B_6-DEFICIENT ADRENALECTOMIZED RATS*

Experiment	*gm.*		*gm. Assumed initial thymus wt.*	*gm. Final thymus wt.*	*gm. Adrenal wt.*	*gm. Thymus weight gain*	*No. of rats*
	I.B.W.	*F.B.W.*					
Adrct., defic.	77.1	82.5	.290	.244	—	−.040	8
Adrct., control	76.6	93.1	.290	.347	—	+.057	12
Intact, defic.	82.5	88.0	.305	.233	.0315	−.072	9
Intact, control	84.0	106.0	.310	.418	.0314	+.108	9

* All animals 1 week on experiment.

TABLE 4

DESOXYPYRIDOXINE ON BODY WEIGHT, THYMUS WEIGHT, AND DERMATITIS IN RATS*

Experiment	*gm.*		*gm. Thymus wt.*	*No. of rats with dermatitis*	*Severity of skin lesions*	*No. of rats*
	I.B.W.	*F.B.W.*				
D.B_6 21 days	181	162	.061	6	++−++++	6
D.B_6 first 10 days	185	174	.097	6	+−++++	6
D.B_6 last 10 days	185	168	.120	4	+−+++	6
Deficient controls 21 days	171	195	.242	0	—	6

* All animals on B_6-deficient diet for 3 weeks.

days after the beginning of the experiment but the feeding of the deficient diet continued, none of the symptoms was alleviated. When the analogue was given only during the last 10 days of the 3-week period of the feeding experiment, the body weight deficit was the same as in the previous groups, while thymic atrophy and dermatitis appeared to be somewhat less pronounced. In rats of the size and age used, the feeding of the pyridoxine-deficient diet alone produced no weight losses or skin manifestations, but a marked deficit in thymus weight occurred. It was seen earlier that the feeding of large quantities of D. B_6 exerts no noticeable effect when adequate amounts of pyridoxine are administered together with the analogue. Following such treatment, however, when both the antivitamin and the vitamin were withdrawn (FIGURE 4), the activity of the antivitamin became ap-

parent weeks after its seemingly ineffective administration had been discontinued. In rats, as in mice (FIGURE 5), quantities of D. B_6 in excess of

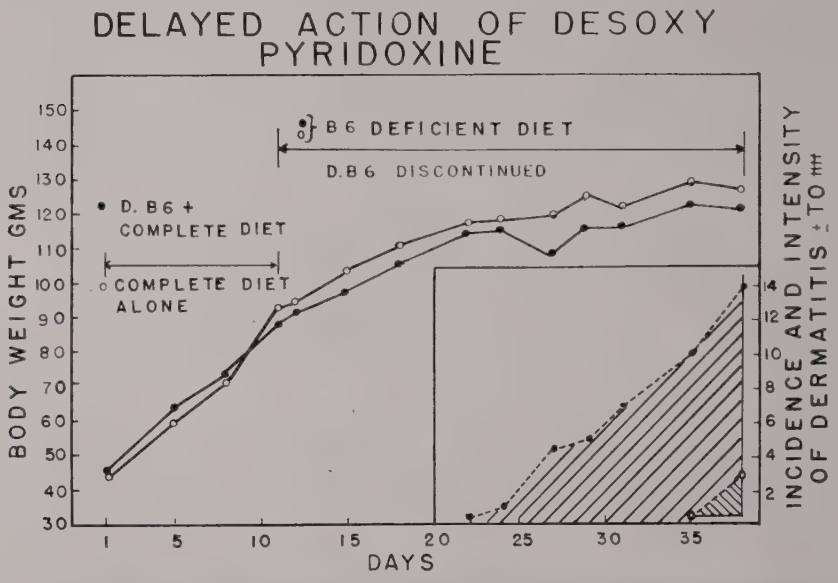

FIGURE 4. Delayed action of desoxypyridoxine. Represents a plot of body weight against number of days on the experiment. The shaded areas correspond to the product of incidence and severity of skin lesions over time. (10 male rats in each group.)

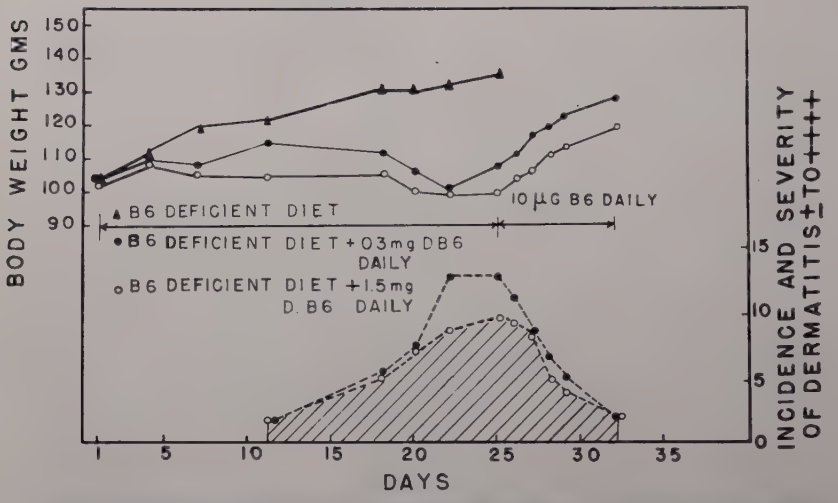

FIGURE 5. Similar plot as in FIGURE 4. (10 male rats in each group.) "Acute pyridoxine deficiency" following the administration of 0.3 and of 1.5 mg. desoxy B^2.

effective doses had no additional influence upon the severity of the symptoms of the "acute deficiency" or on the speed with which they developed.

Also, the curative dose of pyridoxine-HCl was not dependent upon the amount of D. B_6 previously used for producing the disease.

Obviously, no exacting conclusions as to the mechanism of action of D. B_6 can be arrived at from these experiments. When it is tentatively accepted that desoxypyridoxine phosphate represents the actual inhibitor, as proposed by Umbreit, it appears that certain possibilities may be suggested by the above findings. If D. B_6 in the animal is converted into an active form, the quantities in which this principle can be formed appear limited. Its antagonistic effect appears associated with a sparing action upon vitamin B_6 and pyridoxal phosphate. It seems to persist in the body for a considerable length of time. Adequate amounts of pyridoxine-HCl appear to interfere with its action but not with its formation.

From all previous clinical and experimental observations, it appeared that established cancers are characterized by "metabolic autonomy" and persist irrespective of the state of nutrition of the affected organism, and that neoplastic tissue is apparently capable of drawing more readily from the store of nutrients than the normal tissues of the body. Therefore, reviewers of the subject have agreed that no form of "hunger" can be considered as a hopeful means of attack against *established* neoplasms.[16–19] Little is known about the exact mode of action of vitamin antagonists. From circumstantial evidence, however, it appears that they may compete "*in loco*" with their respective vitamins. If such is the case, one may expect that "metabolic autonomy" no longer provides relative protection for the neoplasm from nutritional deficiency. It could even be that higher requirements may render rapidly proliferating tumor tissue even more susceptible to the action of a blocking antagonist. Speculative considerations of this possibility have been expressed,[18, 8, 20] and recently two vitamin antagonists were found to produce drastic effects on neoplastic tissue. It was seen that the administration of D. B_6 was followed by marked regression of lymphosarcoma implants.[12] Also, a folic acid antagonist was shown to be effective against the Rous sarcoma.[21]

The effect of D. B_6 upon lymphosarcoma implants* in C3H mice is illustrated by FIGURE 6. In animals on a complete diet, the tumor reaches an average size of about 10 $cm.^3$ in 3 weeks. In mice rendered acutely deficient by the administration of D. B_6 on a diet devoid of pyridoxine, there was marked regression of the established tumors (FIGURES 7 and 8). "Chronic pyridoxine deficiency" had no such effect. The lymphosarcoma is fast growing and invariably kills within 30 days. No spontaneous regressions have been observed in a large number of observations. Life was prolonged significantly in animals that received intermittent treatment with the antagonist (FIGURE 9). However, complete realimentation with pyridoxine led to recurrence of the tumors and death of the animals. Also, in many experiments, animals with acute pyridoxine deficiency died with diarrhea and emaciation in spite of regression of the tumors. When such mice were autopsied, they showed gross and microscopic evidence of Tyzzer's disease.

* The tumor (6C3H-ED) was obtained through the courtesy of Dr. W. U. Gardner.

This epizootic, although extremely disturbing in the above experiments, appears quite interesting in itself. It will be discussed later in greater detail.

Histologically, the treated tumors showed very striking changes. The untreated lymphosarcoma implants closely resemble the human type of large cell lymphosarcoma (FIGURE 10), showing uniform distribution of tumor lymphocytes with numerous mitoses throughout the neoplasm. Following the administration of D. B_6 (FIGURE 11), the majority of the tumor cells

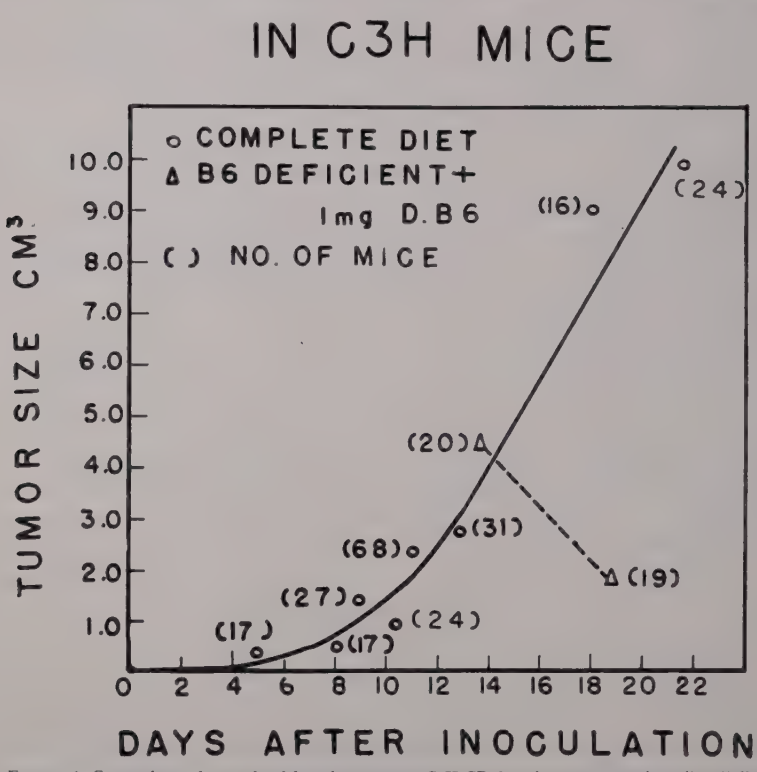

FIGURE 6. Comparison of growth of lymphosarcoma 6C3H-CD in mice on a complete diet (full line with that of the same tumor in mice rendered "acutely" deficient for 5 days (broken line).

exhibit pyknosis, and there appear numerous large, often multinucleated, cells with lobulated hyperchromatic nuclei and abundant cytoplasm. Many of these cells contain phagocytized nuclear debris and not infrequently they resemble "Sternberg cells." Transplants from regressing tumors, in normal mice, regained the morphological appearance of the original lymphosarcoma, although their growth was markedly retarded.

Very recently, Gellhorn and Jones[22] attempted to obtain a therapeutic effect with D. B_6 in patients with neoplasms of lymphoid tissue. Because of the inability of D. B_6 to antagonize the abundance of pyridoxamine present in natural foods,[6] a purified diet had to be fed to the patients. This diet

proved highly unpalatable, and in none of the cases was it possible to continue the regimen for more than 14 days. Three cases of lymphosarcoma were under observation. Measurable regressive changes (up to 50 per cent) were observed in the tumor masses of two of them. This regression was ascribed by the authors to inanition and was compared to the phenomenon of "accidental involution" that occurs in thymic tissue following body weight

FIGURE 7. (a) Mouse #42 before treatment. (b) 5 days after feeding of a diet deficient in vitamin B_6 and 0.9 mg. of D.B_6 daily.

loss from various causes. It seems, however, that no parallelism should be expected to exist between the behavior of normal, juvenile thymic tissue and that of a lymphoid neoplasia. Also, it appears that one could interpret Gellhorn and Jones's findings to indicate that D. B_6, although highly impractical as a therapeutic agent, had sufficient action on human lymphosarcoma to encourage a search for other analogues of the vitamin B_6 group, particularly those that would antagonize pyridoxamine.

With Eisen and John,[10] it was found that immune responses are markedly

impaired in pyridoxine-deficient rats, while deficiencies of *comparable* severity, in other factors of the B complex (thiamine, riboflavin, pantothenic acid) and in protein, had no such effect. It was first thought that the loss of lymphocytes in pyridoxine deficiency accounts for the impairment of antibody responses in this condition. This appeared less probable, however, when it was seen that extreme lymphoid atrophy due to advanced thiamine deficiency was not associated with impairment of immune responses.

It was realized that other nutritional diseases, if sufficiently severe, may

FIGURE 8. (a) Mouse #72 before treatment. (b) After same treatment as in FIGURE 7b.

also suppress antibody formation. However, the fact that comparatively mild pyridoxine deprivation causes marked impairment of antibody formation makes this defect more specific for the lack of vitamin B_6 than for any other nutritional disease.

In this respect, the essential role of vitamin B_6 may be related to any one or several of the phases which lead to the development of the usual immune response. Accordingly, obvious causes could have been: an excessive destruction of the introduced antigen; an incapacity of the antibody forming apparatus (R.E.S.?) to take up antigens; a reduced production of antibody protein; and an excessive destruction of properly formed antibody. These

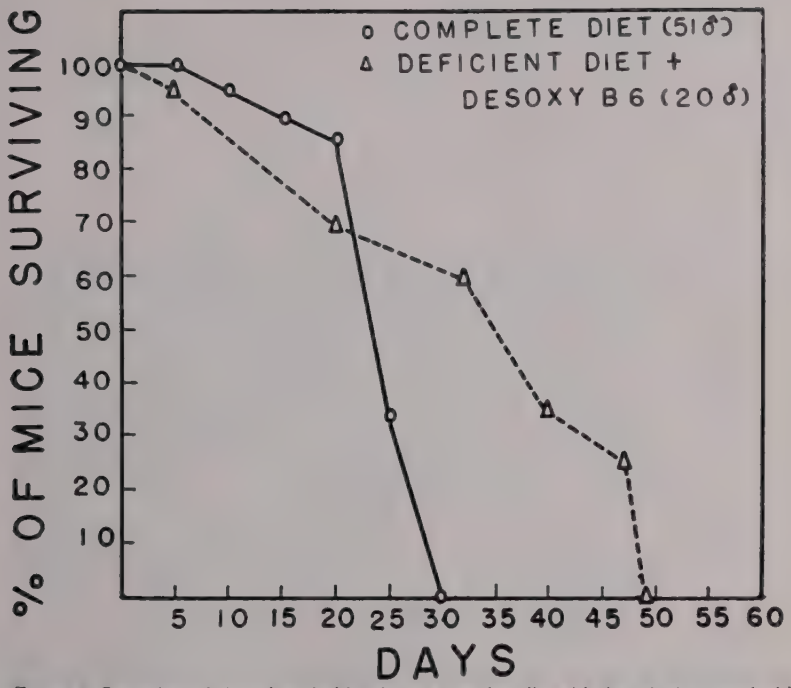

FIGURE 9. Comparison of time of survival in mice on a complete diet with that of mice treated with desoxy B_6. Several courses of treatment over 5 days were given and the animals permitted to recover in intervals of from 3 to 5 days.

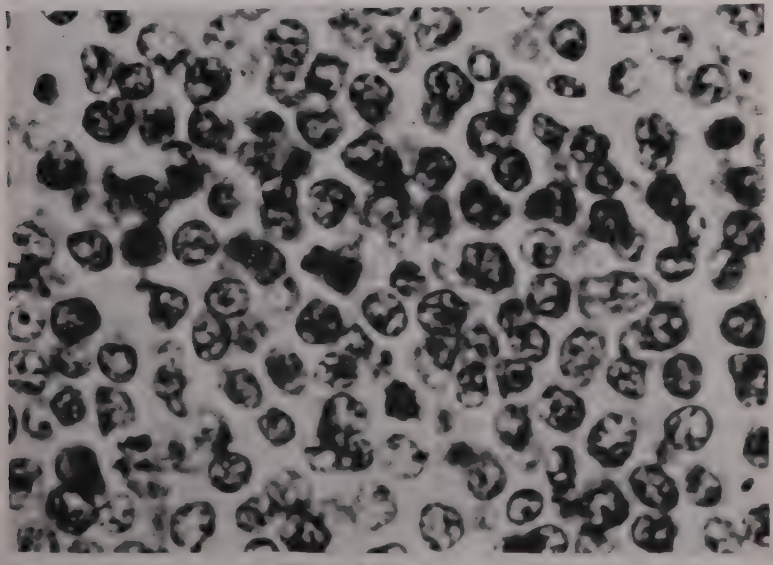

FIGURE 10. Lymphosarcoma 6C3H-ED 450×, showing uniform distribution to tumor lymphocytes throughout the neoplasm. There are several mitotic figures in the field.

possibilities have been explored experimentally in rats by measuring the rate of disappearance of antigenic protein and homologous protein, both labeled with specific antibody (rabbit antisheep and rat antisheep serum). Also, the reticulo-endothelial systems of deficient animals, injected with various dyes and particulate matter, were examined histologically.

Rats with "acute pyridoxine deficiency" (TABLE 5), 5 days after the injection of sheep cells, exhibited lower levels of circulating antibodies than simi-

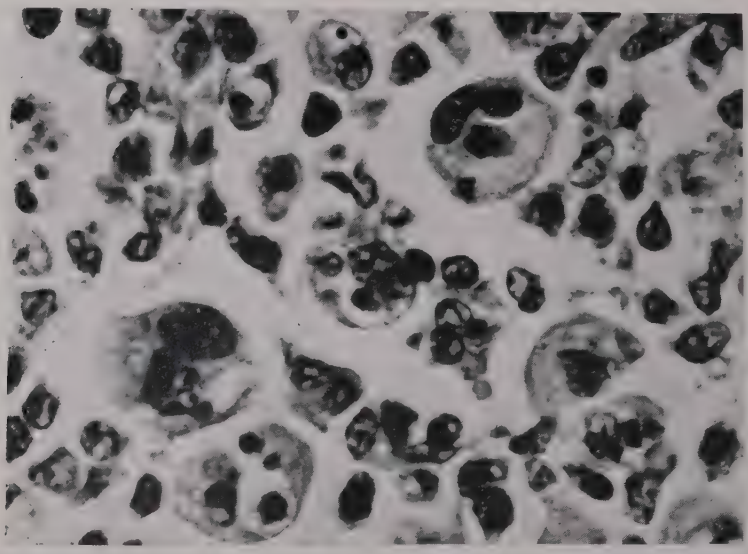

FIGURE 11. Regressing lymphosarcoma (450×). There have appeared large, often multinucleated cells containing phagocytized nuclear fragments. The morphology of the tumor resembles that of a histiocytoma.

TABLE 5

"ACUTE PYRIDOXINE DEFICIENCY" ON THYMUS AND ANTIBODY*

Experiment	*gm.*		*gm. Thymus wt.*	*Agglut. titer, av.*	*Agglut. titer, range*	*No. of rats*
	I.B.W.	*F.B.W.*				
Deficient	171	195	.242	1:320	160–640	6
Deficient plus desoxy B_6	181	162	.061	1:80	0–160	6

* Three weeks on experiment.

larly treated controls. When rats and mice (TABLE 6), immunized 20 weeks before the start of the experiment, were rendered acutely deficient over 3 weeks and then reinjected with the antigen, the usual anamnestic rise failed to occur in the deficient animals. Electrophoretic measurements of serum protein fractions were made by Dr. D. C. Moore from pooled samples of serum of these rats. They failed to reveal a measurable reduction of the gamma globulins or any of the other fractions.

Immunizing rats passively (FIGURE 12) with rabbit antisheep serum and injecting such rats with rat antisheep serum showed that, in pyridoxine deficiency, there is no measurable increase in the breakdown of antigenic protein or of homologous antibody protein. It was also seen that no inhibitory effect was exerted by sera of rats with "acute pyridoxine deficiency" upon

TABLE 6
ANAMNESTIC RESPONSE IN PYRIDOXINE DEFICIENCY*

Experiment		*Hemagglutinin Titers*			*Total no. of animals*
desoxy B_6	B_6	*1 week after first injection*	*20 weeks after first injection*	*1 week after second injection*	
+	+	1:320 (10)	1:16 (20)	1:554 (10)	(48)
+	−	—	—	1:28 (8)	rats
+	+	1:168 (17)	1:114 (8)	1:3213 (8)	(41)
+	−	—	—	1:63 (8)	mice

* All animals on B_6-deficient diet and desoxypyridoxine 3 weeks prior to reinjection of antigen.

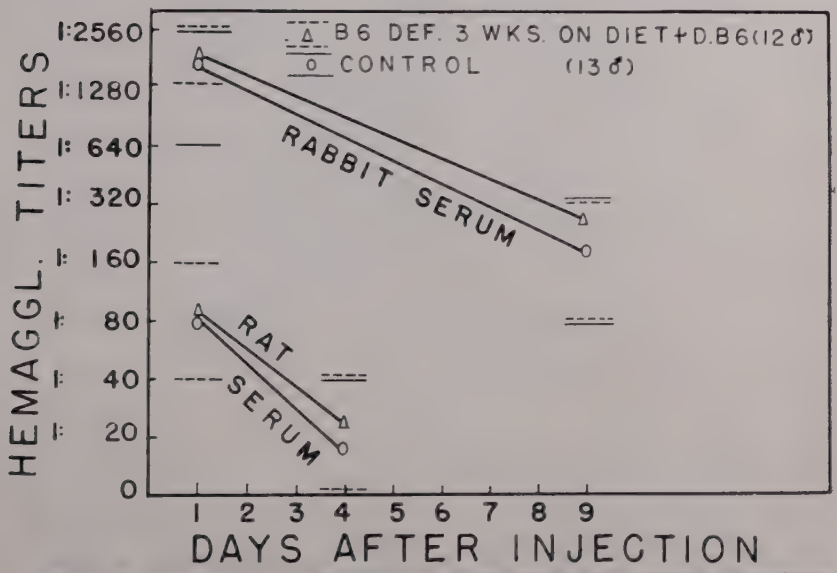

FIGURE 12. Comparison of rate of disappearance of heterologous (rabbit antisheep) and homologous (rat antisheep) antibody from the circulation of normal rats and of rats after 3 weeks of "acute pyridoxine deficiency."

the antigen-antibody reaction measured in these experiments. Valid function tests for the integrity of function of the reticulo-endothelial system have not so far been devised. Qualitatively, the amount of phagocytized injected material in histiocytes may be taken as an indication for the preservation of their activity. By this criterion, the histiocytes of rats with "acute pyridoxine deficiency" appeared normal in this respect.

From these findings, it seems probable that it is the formation of antibody protein which is impaired in pyridoxine deficiency. In support of this possibility, there are several biochemical indications to suggest that the vitamins of the B_6 group are vitally concerned with protein metabolism.

It has been mentioned before that, in many instances, mice of the C3H strain treated with D. B_6 died of Tyzzer's disease. The epizootic was confined to C3H mice with "acute pyridoxine deficiency" and was not seen in even advanced stages of the "chronic deficiency."[11] Very recently, Tyzzer's disease has also been observed in rats rendered "acutely deficient" in pyridoxine.[23] The infection in mice due to "*Bacillus pyliformis*" has been admirably described by Tyzzer.[24] Striking symptoms of the disease were diarrhea and emaciation. At autopsy, the livers of such mice show grossly

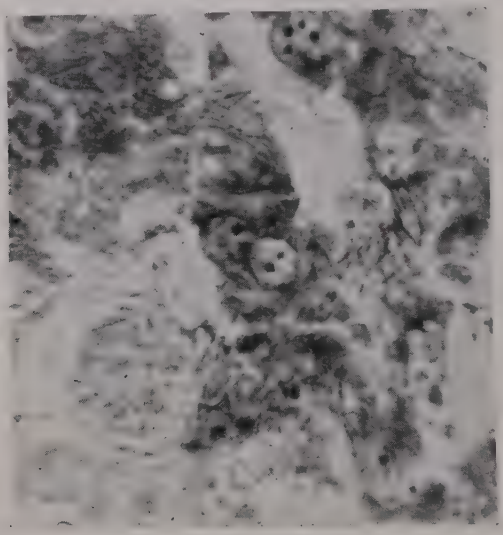

FIGURE 13. High power view of liver of mouse with Tyzzer's disease. The cytoplasm of several liver cells is engorged with bacilli.

fatty infiltration and are studded with opaque, yellowish-gray, rounded nodules of somewhat moist necrotic tissue, up to 2 mm. across. Microscopic examination (FIGURE 13) reveals the presence, in the cytoplasm of liver cells, of elongated, thin, Gram-negative rods, arranged in parallel fashion to intercrossing bundles. They may be found, but do not give rise to gross lesions, in organs other than the liver. Liver cells, engorged with bacillary inclusions, are seen all along the periphery of the necrotic areas.

The interesting feature of the disease is represented by the fact that the susceptibility to this infection was shown to be genetically restricted. It was seen by Tyzzer that only an inbred strain of Japanese waltzing mice and a few hybrids of its first and second generation were affected by the epizootic disease. Other strains of mice were not involved and could not be infected with the organism. The strain of C3H mice used for our experiments was

obtained from Dr. L. C. Strong. It has been under his observation for several decades. Never before the introduction of the "acute pyridoxine deficiency" were these mice found to show this condition.[25] The disease has never been reported in rats.

From these findings, it appears that, in "acute pyridoxine deficiency" surprisingly enough, both innate and acquired resistance are greatly impaired. It should be realized, however, that this is likely to be no more than a strange coincidence. If future investigations should reveal that, in strains of animals susceptible to Tyzzer's disease, there is impairment of the antibody forming mechanism on a genetic basis, a simple explanation for this coincidence will have been found.

Summary and Conclusions

Findings were obtained in pyridoxine deficiency produced by the administration of desoxypyridoxine which are not observed in the nutritional disease that results from feeding of a diet deficient in vitamin B_6 alone.

These characteristics of "acute pyridoxine deficiency" were: (1) a contracture and dermatitis of the tail in rats; (2) a relative increase of the vitamin B_6 content in livers of rats and mice; (3) regression of lymphosarcoma implants in mice; (4) a loss of the ability to respond with an anamnestic rise to the reinjection of an antigen, in mice and rats; and (5) the frequent occurrence of Tyzzer's disease in mice which, under normal circumstances, are resistant against this infection.

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STUDIES ON ANALOGS OF PURINES AND PYRIMIDINES

By George H. Hitchings, Gertrude B. Elion, Elvira A. Falco, Peter B. Russell, and Henry VanderWerff

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A study was begun in these laboratories, in 1942, of the relationship between chemical structure and the ability of certain pyrimidine* derivatives to serve as precursors for or to modify nucleic acid synthesis. Since only brief accounts of small portions of this work have been published to date,[1–4] the present paper is to be regarded as a preliminary report of the work as a whole.

It was felt that such studies might lead to fundamental knowledge of the roles of pyrimidine and purine bases in growth, and of the part played by folic acid in the synthesis of these bases. It was felt also that new chemotherapeutic agents might be discovered by this means since, it was argued, parasitic tissues in general depend for survival on a more rapid growth, hence a more rapid synthesis of nucleic acid, than that of the host tissues. This argument applies equally well to bacterial, viral, rickettsial, and neoplastic diseases, so that, in a sense, one might say we have been searching for the philosopher's stone, the universal panacea, of the ancients.

A distinct advantage of antipurines and antipyrimidines as chemotherapeutic agents seemed to lie in the fact that the requirements of bacteria, at least, appeared to be qualitatively different from those of mammalian tissues. A considerable number of microorganisms[5] were known to require preformed pyrimidines and/or purines for growth, whereas the evidence available at that time indicated that purine and pyrimidine bases played no role in mammalian nucleic acid synthesis.[6] In the interim, of course, it has become known that two purines, adenine[7,8] and 2,6-diaminopurine,[9] do contribute to the nucleic acid purine of mammalian tissues, and the hypothesis becomes subject to modification on this account. The effect of thymine in nutritional macrocytic anemia, sprue, and pernicious anemia[10] indicates that some metabolic role may have to be postulated for this pyrimidine base in the face of the studies with isotopically tagged thymine, which indicated only a catabolic elimination and no retention of exogenous thymine.[6] It is conceivable that guanine, uracil, and cytosine may have metabolic roles which remain undetected in similar studies because of low turnover rates or for other reasons.

The choice of *Lactobacillus casei* as a model biological system for the study of pyrimidine analogs was based on the known requirement of this microorganism for folic acid and the role of thymine and guanine in the satisfaction of this growth requirement.[11] This allows a study of the activity of each substance in a number of different ways in the same microorganism. TABLE 1 shows the six media used for study and the effects of various substances in each of the media. The media contain thymine or

* The term "pyrimidine" is used in its broadest sense throughout this paper to include pyrimidines as such and derivatives of condensed pyrimidine systems such as purines, pteridines, quinazolines, and triazolo[d] pyrimidines.

pteroylglutamic acid (PGA)* alone or in combination with a purine (usually adenine) and the appropriate control media with and without adenine. Three of these media (AFA, BT, BFA) show approximately half-maximal growth, as measured by lactic acid production over a sixty-eight hour period.†‡ The effect of thymine is to stimulate primarily in the blank medium, which contains adenine (BO), somewhat in the medium which contains PGA and adenine (BFA), and not at all in that with PGA but without adenine (AFA). This illustrates the essential nature of the purine requirement for growth with thymine, and the relatively unimportant stimulatory effect which purine has in the presence of PGA. The action of PGA

Table 1

Response of *L. casei* to Various Substances in Different Media

Substance	*Amt./Tube*	*AO*	*AT*	*AFA*	*BO*	*BT*	*BFA*
Control		0.6	0.8	6.0	1.0	6.5	8.0
Thymine	10 γ	0.8	0.8	5.8	6.5	6.6	10.0
PGA	0.46 mγ	6.0	5.8	7.6	8.0	10.0	10.5
Adenine sulfate	100γ	1.0	6.5	8.0	1.0	6.8	8.8
5-Methyl-6-hydroxypyrimidine	1 mg.	0.9	0.9	5.5	7.0	6.5	10.2
6-Methylaminopurine hydrochloride	1 mg.	0.8	3.9	7.0	1.0	6.5	8.1
5-Bromouracil	1 mg.	0.5	0.5	4.8	2.0	2.5	10.0
2,6-Diaminopurine	1 mg.	0.3	0.3	0.8	1.1	6.5	7.0
5-Nitrouracil	1 mg.	0.3	0.6	0.5	0.5	6.0	0.8
5-Aminouracil	1 mg.	0.3	0.5	1.0	0.4	3.3	3.2

A—Medium of Landy, M. & D. M. Dicken, J. Lab. Clin. Med. **27:** 1086 (1942), but with omission of purines, uracil, and folic acid and supplemented or modified as follows (allowances per 100 ml. of medium): *dl*-alanine 20 mg., *dl*-glutamic acid 0.5 mg., $MgSO_4 \cdot 7H_2O$ 40 mg., $FeSO_4 \cdot 7H_2O$ 8 mg., $MnSO_4 \cdot 2H_2O$ 8 mg., pyridoxine hydrochloride 400 γ, *p*-aminobenzoic acid 20 mg., thiamine chloride 20 γ, glucose 1.5 gm., sodium acetate 1.0 gm.

B—Medium A with the addition of 1.0 mg. adenine sulfate per 100 ml.

O—Control media.

T—Addition of 100 γ thymine per 100 ml.

FA—Addition of 4.6 mγ pteroylglutamic acid per 100 ml.

is illustrated on the next line of the table. The addition of a small amount of PGA gives increased growth in all media, but the increase is most marked in the controls AO and BO. The effect of purine is illustrated by adenine sulfate in the next line. The chief effect of a purine is to give growth in the medium which contains thymine but no purine (AT). 5-Methyl-6-hydroxypyrimidine is illustrative of a thymine-like substance and 6-methylaminopurine serves as a substitute for adenine.

The nature of various inhibitory effects also can be seen in these media. Thus, 5-bromouracil appears to be primarily an antagonist of thymine, since growth in the BT medium is strongly inhibited by this substance.

* In early work, a folic acid concentrate of potency 40,000, kindly supplied by Professor R. J. Williams, was used in these media. Although some minor differences between the concentrate and PGA have been detected, the results in the main are essentially the same with either source of folic acid.

† Although lactic acid production is used as the chief criterion of growth in these studies, considerable use is made also of the optical density of cultures and of plate counts. For the most part, these three criteria are in reasonable agreement. However, certain discrepancies have been noted in the text which follows.

‡ In the sixty-six hour period, glycolysis in the thymine medium proceeds to only about half-maximal levels[12,13] and cannot be accelerated by further additions of thymine. Contrary to Stokes (*loc. cit.*), however, glycolysis continues progressively to completion over a 10-12 day period. By increasing the concentration of PGA, on the other hand, growth and glycolysis can be made to go to completion in a period slightly less than sixty-six hours.

2,6-Diaminopurine, at first glance, appears to be a PGA antagonist, since it strongly inhibits folic acid growth (AFA). Its effects are seen to be easily reversed by adenine (BFA), however, and reversal studies (described later, FIGURE 23) leave no doubt that it acts to interfere primarily with adenine metabolism in this biological system. 5-Nitrouracil is more characteristic of the "antifolics." Its action in the inhibition of folic acid growth (AFA and BFA) duplicates that of 4-amino-folic acid and other antagonists built on the PGA model. 5-Aminouracil inhibits in all media (AFA, BT, BFA) and, as will be shown (FIGURES 1 and 2), can be reversed by either thymine or PGA.

It is commonly assumed in antimetabolite studies that a growing culture of a microorganism can be treated as a single enzyme system, *i.e.*, a single

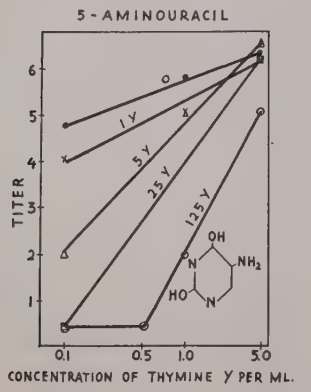


FIG. 1

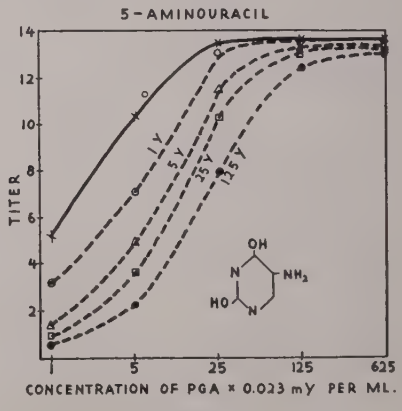


FIG. 2

FIGURE 1. Antagonism of thymine by 5-aminouracil.
FIGURE 2. Antagonism of PGA by 5-aminouracil.

enzyme is assumed to become the limiting factor for growth in the presence of a substance which specifically interferes with its action. Many types of antagonisms are known[14] and the chief problem is to sort out those more or less intimately concerned with specific enzyme systems. The best available test is the reversal experiment, in which a competitive inhibition between metabolite and antimetabolite can be demonstrated. Given a competitive inhibition with a ratio of metabolite and antagonist which will produce equivalent effects at several concentrations, one feels reasonably sure that he is dealing with exchange of one substance for the other on some cell receptor. In the absence of direct information, however, it should not be assumed that the metabolite in question is a prosthetic group of an enzyme and that competitive exchanges necessarily occur only on enzyme surfaces, for many other possibilities exist.[15]

To be thoroughly convinced of the competitive nature of an inhibition, one would like to be able to show not only (1) constant effects at a constant ratio of inhibitor to metabolite, but also (2) complete restoration of growth with an excess of metabolite, and (3) complete suppression of growth with

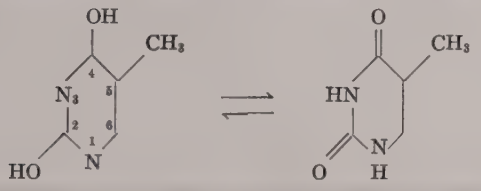
an excess of antimetabolite. The number of inhibitors capable of satisfying these criteria is very limited. In a search for antagonists, one finds a considerable number of substances which act somewhat, but not quite, as one thinks they should, and there is a great temptation to gloss over the effects and to draw conclusions which perhaps are unwarranted. Some of the inviting pitfalls which are open to the unwary investigator can be illustrated in the studies to be described.

The Thymine Requirement

The first objective of the present studies was to determine the specificity of the thymine molecule for the growth of *L. casei*, *i.e.*, which chemical

TABLE 2

EFFECTS OF ANALOGS OF THYMINE ON *L. casei*



Thymine (5-methyluracil)

Substitutes	*Inhibitors*
2-H	2-SH
2-NH_2	2,4-di SH
4-NH_2	2,4 di NH_2
2,4-di NH_2	5-OH
1-Me	5-NH_2
3-Me	5-Br
5-Ethyl	5-NO_2
5,6-glycol	

groupings were essential and acceptable to the cell receptors. TABLE 2 shows the results of some of these studies. In the first column are listed some of the substances which may serve as substitutes for thymine. With the exception of thymine glycol, the thymine substitutes are less active than thymine in that higher concentrations are required to give equivalent growth effects. The activity of the amino compounds is especially interesting. The substitution of an amino for a hydroxyl group diminishes the activity by a factor of about 10. Thus, the two monoamino-monohydroxy-5-methylpyrimidines are able to substitute for thymine at about ten times the usual thymine concentration, and the 2,4-diamino-5-methylpyrimidine is about 1/100th as active as thymine. The diamino derivative is especially interesting because it can serve as a thymine substitute when tested for thymine-like activity, yet it also has inhibitory activity when tested on folic acid growth in the absence of adenine (AFA). The latter property is common to all 2,4-diaminopyrimidine derivatives.

The activities of the thymine substitutes may be interpreted in either of two ways.* It can be assumed that the substances are transformed to thymine, in which case a considerable variety of metabolic transformations have to be postulated (deamination, demethylation, reduction). Alternatively, it might be assumed that the substances act without modification. As a corollary to this hypothesis, the formation of a desoxyribonucleic acid containing an abnormal basic constituent would probably have to be postulated. Somewhat in favor of direct utilization of some of these substances is the finding that certain combinations of the methylcytosines actually give better growth than that obtainable with thymine. A definitive answer to these problems probably could be obtained by experiments with isotopically labeled bases.

Thiothymine[16] and dithiothymine act as thymine antagonists. The most interesting group of inhibitors of *L. casei*, however, are those which may be viewed as thymines in which the 5-methyl group has been replaced by a different group. It had been expected that such modifications of the thymine molecule would produce antithymines. Actually, one can distinguish at least four types of inhibitors among such substances—types represented here by 5-hydroxyuracil (isobarbituric acid), 5-aminouracil, 5-bromouracil, and 5-nitrouracil.

5-Aminouracil inhibits the growth of *L. casei* in the presence of either thymine or PGA. FIGURE 1 shows the results of reversal experiments involving thymine and 5-aminouracil. Increasing concentrations of 5-aminouracil progressively decrease the growth response, and increases in the thymine concentration act to restore growth. However, this inhibition is not competitive. Complete restoration of growth is attained at about the same thymine concentration regardless of the concentration of inhibitor.

FIGURE 2 shows the relationship between 5-aminouracil and PGA. This is not a competitive inhibition either. To produce equivalent suppression of growth at different concentrations, the aminouracil must be varied as an exponential function of the PGA concentration.

The role of 5-hydroxyuracil (isobarbituric acid) was puzzling at first, since it was found that growth with thymine and with PGA were suppressed about equally by this substance but that reversal occurred with neither. Somewhat later, a study of the role of uracil in the growth of *L. casei* was undertaken, and it was discovered that isobarbituric acid behaves as a uracil antagonist. FIGURE 3 demonstrates the reversal of the inhibitory effect of isobarbituric acid by uracil in both PGA (BFA) and thymine (BT) media. In both media, the anti-uracil effect of isobarbituric acid is competitive, essentially constant inhibition ratios being obtained. This draws attention to the fact that uracil can be synthesized by the organism in the absence of PGA. Moreover, there is no apparent difference in this mechanism in the organisms grown with thymine as contrasted with those grown with PGA.

* The possibility that certain of the less active substances, such as 2,4-diamino-5-methylpyrimidine, might contain sufficient thymine (*e.g.*, 1 per cent) to account for the growth-promoting activity cannot be completely excluded. This cannot account for the effects of the more active substances (*e.g.*, 5-methylcytosine, 5-methylisocytosine), where the biological effect would require the impurity to be of the order of 10 per cent.

The third type of inhibitor among those formally related to thymine by replacement of the 5-methyl group may be represented by 5-bromouracil (5-chlorouracil and 5-iodouracil give similar results). In FIGURE 4 are shown the results of experiments designed to demonstrate the relationship between bromouracil and thymine. It is seen that at a ratio of about 250 parts of bromouracil to 1 of thymine (molecular ratio about 165:1) nearly complete suppression of growth occurs, and that this ratio produces equivalent effects over a considerable concentration range. Bromouracil and thymine therefore provide a good example of competitive inhibition.

In contrast to the effect on thymine growth, bromouracil has no suppressive effect on growth with PGA. As a matter of fact, a reproducible slight stimulation is observed. Perhaps the simplest explanation of these observations is that thymine, as such, is not produced during growth with

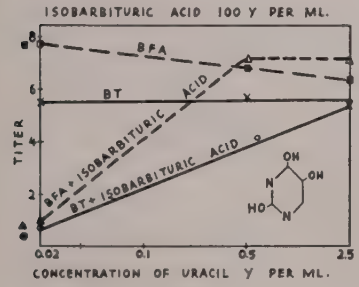


FIG. 3

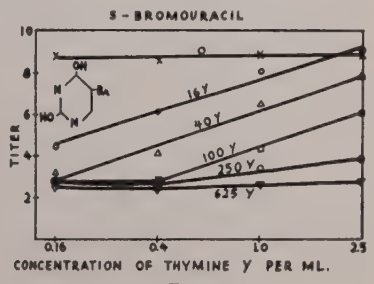


FIG. 4

FIGURE 3. Isobarbituric acid as a uracil antagonist.
FIGURE 4. 5-Bromouracil as a thymine antagonist.

PGA,[1] but that the thymine nucleus is built up on a basic glycoside structure, whether directly in the nucleic acid molecule or in a nucleotide or nucleoside.

Alternatively, it might be assumed that, when the organisms are grown with PGA, thymine is produced in concentrations sufficiently high to nullify the effects of bromouracil. The concentrations which would be required would appear to be unreasonably high, but it can be argued[17] that a metabolite produced intracellularly can be much more effective than the same metabolite when added to the external medium. This and other alternative explanations[17] for the different action of bromouracil in thymine and PGA media are not very useful, since they involve assumptions which are not susceptible to experimental verification.

The fourth type of antagonist, based on the modification of the 5-position of the thymine molecule, may be represented by 5-nitrouracil. This substance, in direct contrast to 5-bromouracil, has little effect on the growth of *L. casei* with thymine, but suppresses growth with PGA in proportion to the concentration and, as can be seen from FIGURE 5, acts as a competitive antagonist of PGA. Over a considerable range, a constant inhibition ratio is obtained.

The activity of nitrouracil in inhibiting growth with PGA and its failure to do so when the organisms are grown with thymine confirm the impression

suggested by the bromouracil studies, that the metabolic pathways of the organism depend in some respects on the nutrilite available for growth.

It was found in early work (FIGURE 5) that a reversal of the effect of nitrouracil could be obtained with thymine. This suggested that nitrouracil might inhibit by blocking the function of PGA which deals with the synthesis of thymine but not that dealing with the synthesis of purine. Addition of thymine to a system containing PGA and nitrouracil would, according to this hypothesis, allow restoration of growth, since purine would be available from the action of the PGA-enzyme system. It is untenable, however, in this instance at least, for two reasons. In the first place, the growth characteristics (acid production in sixty-six hours) in a medium containing thymine, nitruracil, and PGA are not those of the medium

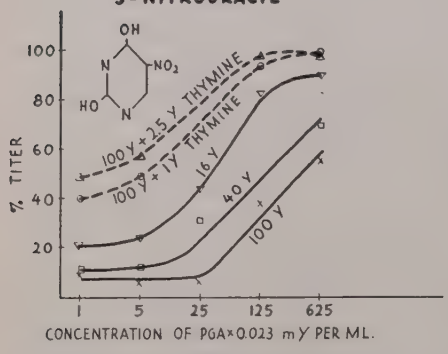


FIG. 5

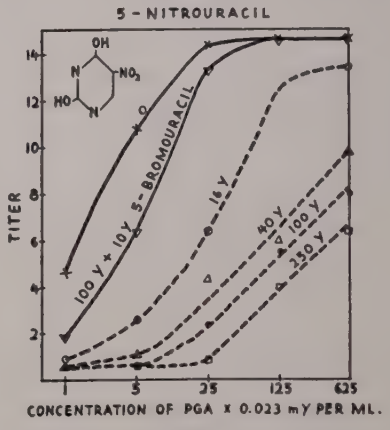


FIG. 6

FIGURE 5. 5-Nitrouracil as a PGA antagonist—reversal by thymine.
FIGURE 6. Effect of bromouracil on inhibition by nitrouracil.

containing thymine and purine but those of the medium containing PGA. In the second place, considerable doubt is thrown on this explanation by the finding that bromouracil behaves in much the same manner as thymine. In FIGURE 6, it is seen that the inhibition of growth produced by 100 micrograms of nitrouracil is nearly abolished when 10 micrograms of bromouracil also are present (comparable results are obtained with 2.5 micrograms of thymine).

No completely satisfactory explanation of this effect of bromouracil suggests itself. Any plausible chemical reactions of either bromouracil or nitrouracil (hydrolysis, reduction) with other constituents of the medium would lead to inhibitors (isobarbituric acid, aminouracil) of known potentialities. Interaction of bromo- and nitrouracils can be ruled out, since nitrouracil has no effect on the inhibition by bromouracil of thymine growth. The best explanation, though not very concrete, may lie in the suggestion that both substances act on the surface of the cell and do not enter the enzyme systems at all. If so, one must assume that some difference exists between the cell surfaces of thymine-grown and PGA-grown organisms.

(Some confirmatory evidence for this is obtained from studies of the glycolysis of cell-suspensions in the Warburg.[18]) Perhaps more important is the implication which also would follow that an apparently clear-cut competitive inhibition may be primarily a cell-surface phenomenon, not involving the cell-enzyme systems at all.

The Purine Requirement

The purine requirement of *L. casei* is an obligate requirement when the organisms are grown with thymine. The purine requirement can be satisfied equally well by adenine, guanine, hypoxanthine, and xanthine. This indicates the presence of biochemical mechanisms for the interconversion of adenine and guanine and for the conversion of xanthine and hypoxanthine into the aminopurines. Certain methylpurines, such as 1-methylguanine,

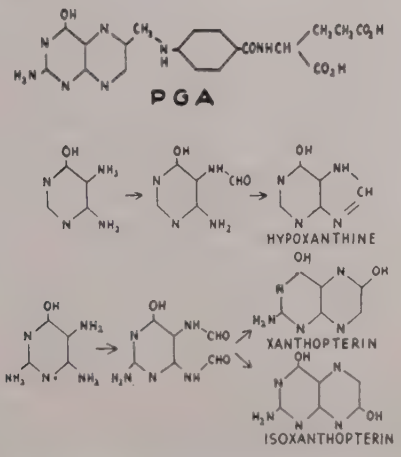


FIGURE 7. Possible role of PGA in the synthesis of purines and pteridines.

also are utilized. This indicates the ability either to demethylate or to use the substance as such.* The finding that certain 5-formamido-4-aminopyrimidines appear to support growth,† whereas the 4,5-diaminopyrimidines are inactive, suggests the possibility that the organism may be able to effect closure of the pyrimidine ring providing the constituent atoms all are available.

Some confirmation of a metabolic role for 5-formamidopyrimidines may be derived from the observation that certain 5-acylamidopyrimidines are strong inhibitors of growth (unpublished experiments). This suggests that the organism, in the absence of PGA, has the ability to close the imidazole ring of the purine providing the constituent atoms are all available, but that the ability to formylate a diaminopyrimidine under these circumstances is lacking. Therefore, one might picture the biosynthesis of purine as follow-

* The ability of 1-methylguanine to replace guanine in certain biochemical mechanisms has been noted previously.[19]

† The activities of these substances are of the order of 1 per cent of the corresponding purines. The possible presence of the required amount of purine as an impurity cannot be rigidly excluded.

ing essentially the route used by Traube,[20,21] synthesis and formylation of 4,5-diaminopyrimidines. The role of the PGA-enzyme system would then be that of formylation in the 5-position of the pyrimidine to supply the carbon atom in position 8 of the purine ring (FIGURE 7). Pteridines might arise by diformylation of similar diaminopyrimidines followed by a pinacol type reaction and dehydration. Some chemical precedents for this reaction also exist.[22]

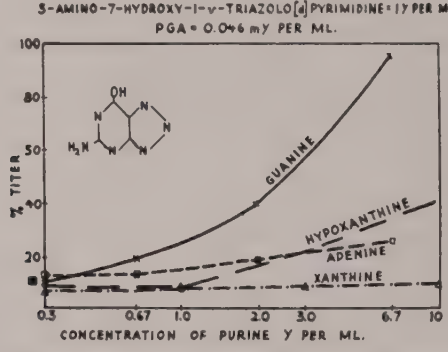


FIGURE 8. Effects of purines on inhibition by aminohydroxytriazolopyrimidine.

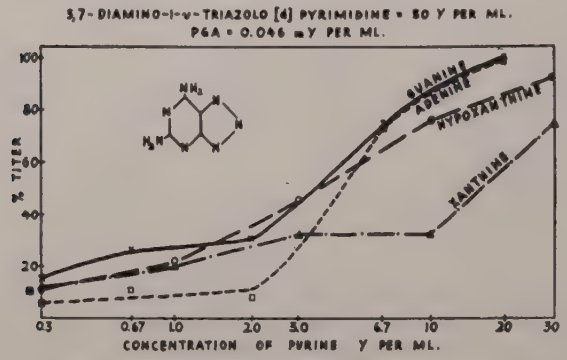


FIGURE 9. Diaminotriazolopyrimidine as a purine antagonist.

It should be emphasized that no direct evidence whatever exists for this view of the role of the PGA enzyme system, and it is felt that isolation of the enzyme system will be a prerequisite for the performance of a definitive experiment.*

When the search for purine antagonists was begun, it was expected that the effects of such substances would be detected primarily during thymine growth (TABLE 1, BT medium), where purine is an obligate requirement.

* The suggestion has been made[23] that PGA functions as a formylating system for the synthesis of purine from 4(5)-aminoimidazole-5(4)-carboxamide, in which case the carbon atom introduced by the PGA system would enter the 2-position of the purine ring. Recent work by Greenberg[35] with pigeon liver homogenate suggests that ribosidization precedes the closure of either ring and that, in this system, the imidazole ring is completed before closure of the pyrimidine ring.

In every instance, however, the effects of such inhibitors are seen primarily in the medium containing folic acid (AFA, TABLE 1). The reason for this is not completely clear. Usually the inhibition, percentage wise, is less in the thymine medium and may be negligible. When inhibition is shown, however, about the same concentration of purine is required for complete reversal in both media.

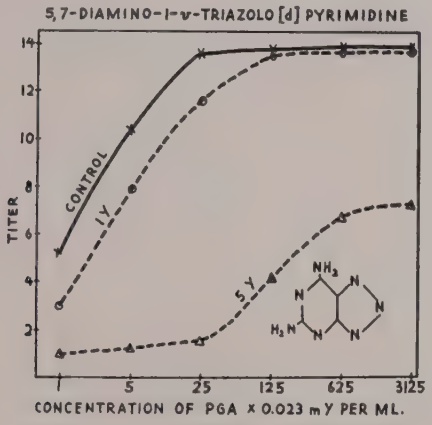


FIGURE 10. Effect of PGA on inhibition by diaminotriazolopyrimidine

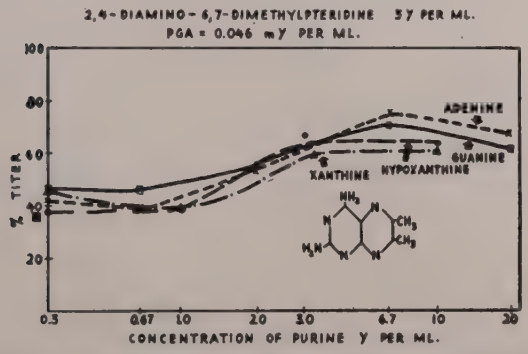


FIGURE 11. Effect of purines on inhibition by diaminodimethylpteridine.

FIGURE 8 shows the effects of a guanine analog[24] on growth in a medium containing PGA and the reversal of the inhibition by various purines. In agreement with the studies of Roblin and coworkers on *Escherichia coli*,[24] this substance inhibits growth, and the inhibitor is more or less a specific guanine antagonist. Some, but definitely less, inhibition is shown in the thymine medium (guanine 0.67 γ, titer 52 per cent; guanine 2.0 γ, titer 76 per cent), and here again guanine is considerably superior to adenine and other purines in the restoration of growth.

FIGURE 9 shows the results of similar studies with a closely related substance, the diamino analog. This substance, in common with the dihydroxy

derivative,[24] shows little specificity for individual purines. FIGURE 10 shows the results of attempts to reverse the action of this substance by increasing the concentration of PGA. It is seen that some increased growth is obtained with the higher PGA concentrations, but full growth is not restored even though the inhibitor concentration in this experiment (5γ) is only one-tenth that of the previous experiment.

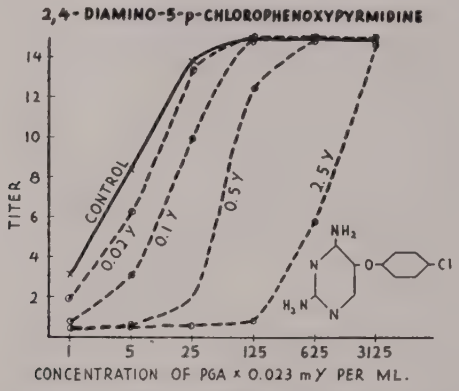


FIGURE 12. Effect of PGA on inhibition by diaminodimethylpteridine.

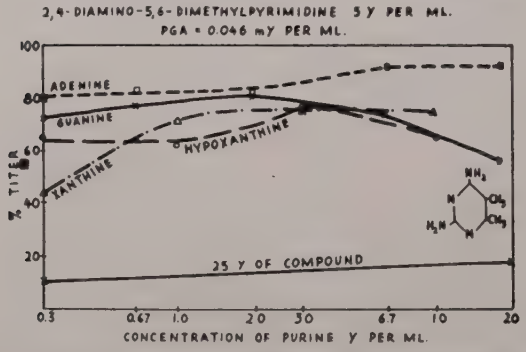


FIGURE 13. Effect of purines on inhibition by diaminodimethylpyrimidine.

This diamino derivative is a representative of a considerable series of substances which may be regarded as derivatives of 2,4-diaminopyrimidine. Every such substance which has been tested has been found to be a strong inhibitor of growth (in AFA medium). However, reversal experiments tend to classify such substances as being primarily antagonists of either PGA or purine, and in some instances a high degree of specificity for individual purines may be shown.

FIGURES 9 and 10 showed that the inhibitions of the diaminotriazolo compound could be reversed by purines and not by PGA. The opposite effect is shown by the 2,4-diamino-6,7-dimethylpteridine, which was studied by Daniel, Norris, Scott, and Heuser.[25] FIGURE 11 shows that addition of

purine has only a little effect on the inhibitory action of this substance even at relatively low degrees of inhibition (control value shown by the square in the vertical margin), and no specificity of the individual purines is shown. However, the inhibitions are readily reversed by PGA at much higher concentrations (FIGURE 12). The inhibition ratio is not strictly constant,

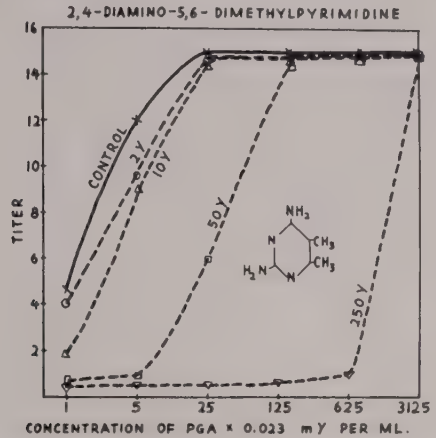


FIGURE 14. Antagonism between PGA and diaminodimethylpyrimidine

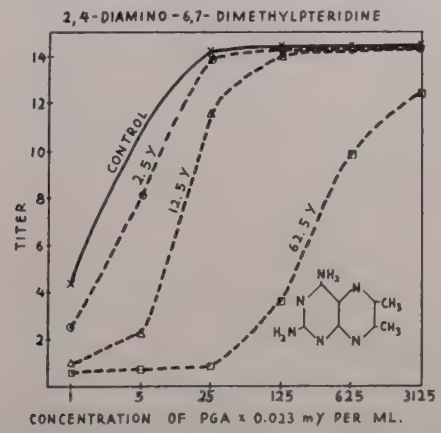


FIGURE 15. Effect of PGA on inhibition by phenoxydiaminopyrimidine.

although the data have been so interpreted,[25] and at high concentrations, full restoration of growth does not occur.

Quite similar to the pteridine is the 2,4-diamino-5,6-dimethylpyrimidine. FIGURE 13 shows that it is even less responsive to purine than the pteridine, while FIGURE 14 shows that the reversal by PGA extends over a wider range of concentration. The inhibition ratio (for complete suppression of growth) is more nearly constant, but again falls off at higher

inhibitor concentrations even though, with this substance, in contrast to the pteridine, full restoration of growth can be obtained.

Another diaminopyrimidine derivative of considerable interest is the 2,4-diamino-5-*p*-chlorophenoxypyrimidine shown in FIGURE 15. This compound is of particular interest because it illustrates how different the apparent behavior of a substance may be in two biological systems. This substance is seen in tests with *L. casei* (FIGURE 15) to be a rather potent antagonist of PGA. Its action is not reversible by purine, and in general it gives the impression of being an "anti-folic" of considerable potency. Dr. George W. Kidder has kindly allowed mention to be made of his unpublished experiments with this compound in *Tetrahymena gelii*.[26] In *Tetrahymena*, which can be grown only in the presence of a rather high concentration of PGA,[27] this compound is highly inhibitory and specifically

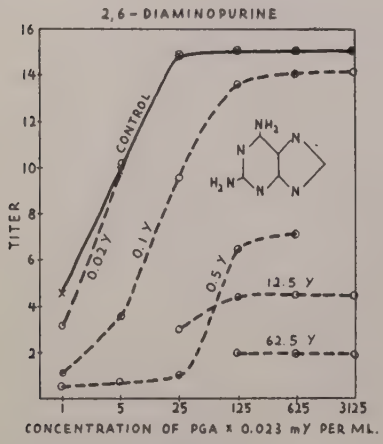


FIG. 16

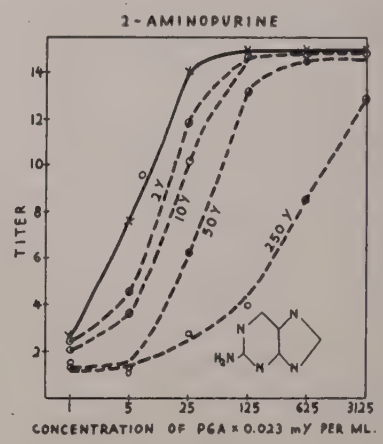


FIG. 17

FIGURE 16. Effect of PGA on inhibition by diaminopurine.
FIGURE 17. Reversal of inhibition of 2-aminopurine by PGA.

reversed by uracil, with the lowest inhibition index of any of a number of substances tested for anti-uracil action.[28] Only a slight tendency toward reversal by uracil is shown with *L. casei*.

Among the diaminopyrimidine derivatives, a number have been discussed which give the appearance of PGA antagonists in reversal experiments. The opposite end of the spectrum may be illustrated by diaminopurine, which produces strong inhibitions reversible by purines specifically and by adenine in particular when the concentration of inhibitor is high. FIGURE 16 shows the results of experiments designed to test reversal with PGA. It is seen that concentrations as low as 0.1 γ per ml. produce inhibitions and that restoration of growth is not complete at any concentration of PGA.

2-Aminopurine (FIGURE 17), on the other hand, does show a tendency toward reversal by PGA, but not as a competitive inhibitor. Since its action also is reversible by purines, its biological behavior is like that of certain diaminopyrimidine derivatives which are intermediate in action

between 2,4-diamino-5,6-dimethylpyrimidine (FIGURES 13 and 14) and 2,6-diaminopurine (FIGURES 16 and 21).

When 2-aminopurine was first investigated,[29] no inhibition whatever was seen in the thymine medium (BT). This is illustrated in FIGURE 18, which shows the growth of *L. casei* with thymine and 1/10th the usual concentration of adenine over a fourteen-day period. It is seen that at the usual assay time (3 days) no significant inhibition appears, even in the presence of a restricted amount of adenine, but that an inhibition does become ap-

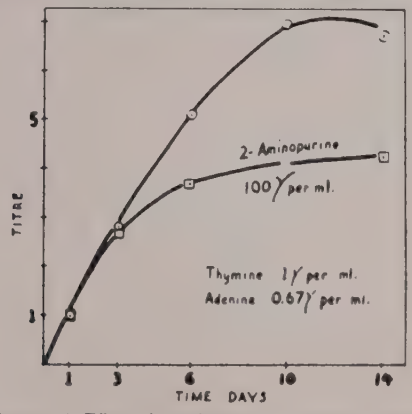


FIGURE 18. Effect of 2-aminopurine in thymine growth.

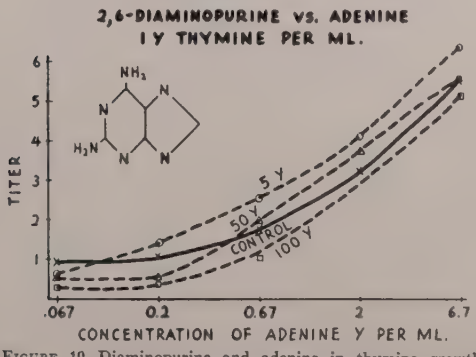


FIGURE 19. Diaminopurine and adenine in thymine growth.

parent under these conditions if the lactic acid production is studied over an extended period of time.

Diaminopurine also shows little tendency toward inhibition of thymine growth. In FIGURE 19 are given data for diaminopurine in the presence of thymine and varying concentrations of adenine. Actually, at the lower levels of concentration, some stimulation rather than inhibition appears to occur. When guanine, however, is used as the source of purine (FIGURE 20), inhibition is seen, and at a concentration of 100 γ of diaminopurine per ml. the inhibition of growth is nearly complete.

The contrasting activities of the different purines in effecting reversal are brought out more clearly in studies of growth with PGA. FIGURE 21 shows the results of reversal experiments at a low concentration of diaminopurine, and it is seen that adenine, guanine, and hypoxanthine are about equally effective in the restoration of growth. The same is true of 2-aminopurine even at very high concentrations of the inhibitor (FIGURE 22). However, at higher concentrations of diaminopurine (FIGURE 23), adenine brings

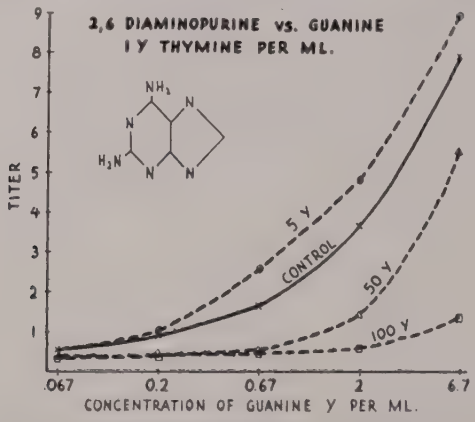


FIGURE 20. Diaminopurine and guanine in thymine growth.

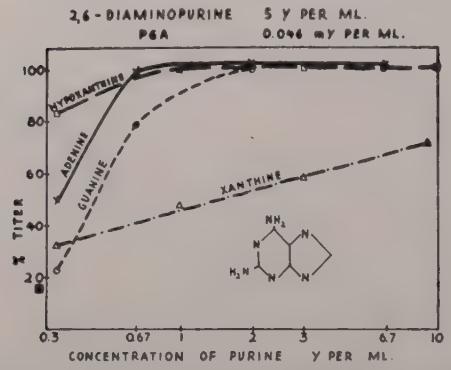


FIGURE 21. Reversal of inhibition by diaminopurine (low concentration).

about complete restoration of growth, while the effect of other purines is negligible. The relationship is not that of a competitive inhibitor of adenine. Perhaps the best way to summarize this effect is simply to say that, in the presence of 2,6-diaminopurine, *Lactobacillus casei* acquires a specific adenine requirement. This could mean that diaminopurine interferes with the synthesis of adenine by a PGA-containing enzyme. Since guanine is unable to supply the deficiency even in the thymine medium, however, one must assume that the transformation of guanine to adenine also is blocked. Actually, the data suggest that the interference with the utiliza-

tion of exogenous adenine is minimal as compared with that of adenine (or its equivalent) when synthesized intracellularly.

The effects of diaminopurine in mammals, the mouse,[30] rat,[30,31] dog,[30] pig,[31] man,[32] and in the chick[33] are about what one would expect of a substance which interferes with nucleic acid metabolism in some way. These include profound bone marrow changes, which, however, can be distinguished from those produced by "anti-folics" such as 4-aminopteroylglutamic acid ("Aminopterin").[30,31] The activity of 2,6-diaminopurine in

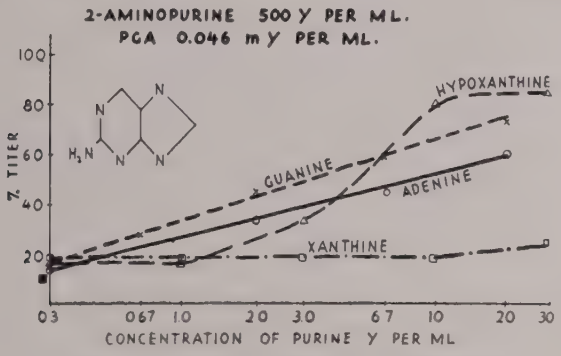


FIGURE 22. Reversal of inhibition by 2-aminopurine.

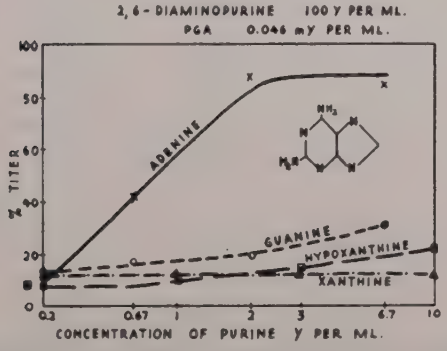


FIGURE 23. Reversal of inhibition by diaminopurine (high concentration).

experimental leukemia[34] and against vaccina virus in tissue culture[35] fits into this pattern of biological activity.

One further fact concerning diaminopurine must be considered in any explanation of its activity. That is the conversion of diaminopurine into nucleic acid guanine as observed by Bendich and Brown.[9] In line with this observation is the finding of Kidder[28] that diaminopurine serves as a (poor) source of purine for *Tetrahymena*, which, it will be remembered, has a guanine but no adenine requirement.

Brown and coworkers[7,8] have observed the conversion of exogenous adenine into adenine and guanine of the nucleic acids and have confirmed

the report of Plentl and Schoenheimer[7] that isotopically labeled guanine is not incorporated into the nucleic acids. Since free guanine does not enter the nucleic acid, the appearance of the labeled nitrogen of diaminopurine in the guanine fraction of the nucleic acid indicates that diaminopurine must exist as a nucleoside, nucleotide, or in the nucleic acid molecule before the conversion can occur. It is possible that the interference with adenine metabolism occurs in one of these stages rather than with the free base. The multiplicity of naturally occurring substances which contain adenine makes interpretation exceedingly difficult.

Conclusions

It may be concluded that pyrimidine derivatives can be found which do interfere with nuclear synthesis and metabolism in a variety of ways. In most instances, the antimetabolite type of experiment is capable of interpretation in several different ways with equal logic, due primarily to the insufficiency of knowledge concerning the normal biochemical mechanisms involved. However, work with antagonists is capable of suggesting the possibility of biochemical reactions which would not readily have been discovered by other means.

In particular, the current work indicates an intimate connection between pteroylglutamic acid and nucleic acid metabolism which is of a considerable complexity. The universality of the biological activity of diaminopyrimidine derivatives suggests the existence of cell receptors for this type of substance and the probability that one or more substances of this kind are involved in normal metabolic pathways.

Finally, some qualitative differences in the activity of various analogs in different biological systems have been brought out and more may be expected from extensions of these studies to other tissues. The existence of qualitative differences in the biochemical patterns of nucleic acid synthesis in different cells, which is suggested by differences in nutritional requirements, is thus confirmed and extended. These qualitative differences may be expected to provide a rational basis for the synthesis of new and useful chemotherapeutic agents.

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PTEROYLGLUTAMIC ACID ANTAGONISTS

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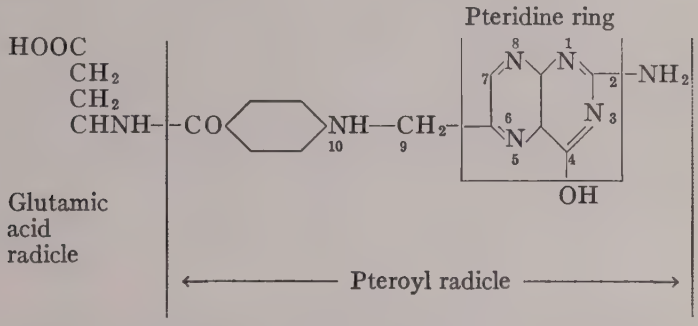
Early studies with a deficiency of "folic acid" in experimental animals led to the production of effects which indicated a relation of this vitamin to cytopoiesis. Monkeys on deficient diets were found to develop a megaloblastic anemia[1] and a severe cytopenia,[2] while the deficiency in chicks[3] produced macrocytic anemia. In rats, leucopenia, granulocytopenia, and hypoplasia of the bone marrow were described by Spicer and co-workers.[4] Studies in bacterial nutrition[5,6] indicated an interchangeability between folic acid and thymine, thus perhaps suggesting a role for folic acid in the formation of nucleic acid and further emphasizing the role of this vitamin in cellular proliferation. The discovery of the structure of the molecule of pteroylglutamic acid (PGA)[7] made it possible to devise and execute methods for synthesizing analogous compounds in an effort to obtain substances which would be biological antagonists of PGA.

The first preparation of this type to be described was made by condensing 2,4,5-triamino-6-hydroxypyrimidine with dibrombutyraldehyde and D-glutamic acid and was termed "7-methyl folic acid." It was reported to be a "displacing agent" for PGA in tests with lactic acid bacteria.[8] Independently, a similar product was made by the same type of condensation except that L-glutamic acid was used, and the resulting preparation, crude "x-methyl" PGA, was studied with lactic acid bacteria, rats,[9] mice, and chicks.[10] The product was found to be an antagonist for PGA in the microbiological assay and to produce a deficiency accompanied by leucopenia, agranulocytosis, and anemia with hypoplastic bone marrow changes in rats and by reductions in the red and white blood cell counts in mice and chicks. All the manifestations were prevented or reversed by increasing the dietary level of PGA to an appropriate height. The activity of the antagonist in depressing cytopoiesis, together with the easy reversibility of this activity by PGA, led to the suggestion[9] that crude "x-methyl" PGA might be used in an attempt to modify blood dyscrasias which were marked by erythrocytosis or leucocytosis. Clinical experiments with the preparation indicated that it was of quite low potency for human subjects as judged by its effects on a patient with chronic myeloid leukemia.[11] The observation was made, however, that the administration of PGA to three patients with chronic myeloid leukemia was attended by relapse, suggesting that further investigations with PGA antagonists should be made.

A PGA analog, pteroylaspartic acid, was synthesized and studied by Hutchings and co-workers.[12] It was found to be a reversible antagonist for PGA in assays with *Lactobacillus casei* and *Streptococcus faecalis* R. Pteroic acid, pteroyl-gammaglutamyl-glutamic acid, and pteroyl-gammaglutamyl-gammaglutamyl-glutamic acid were found to reverse the inhibitory effect of pteroylaspartic acid for *S. faecalis* R. Pteroylaspartic acid was found to antagonize PGA as a dietary essential for chicks in a ratio of 500 to 1, but no effects were observed in experiments with rats.

Other compounds which are antagonists of PGA are exemplified in TABLE 1. The molecule of PGA is numbered as follows to facilitate the naming of analogs:



Crude "x-methyl" PGA is obviously a mixture, but its biological effects are readily reversed by PGA, and further studies with this crude antagonist have been made in a variety of species. Effects on pigs were described by Welch, Heinle, and co-workers[22] and by Cartwright, Wintrobe, and co-workers.[23] Severe anemia and bone marrow changes were noted, together with diarrhea and loss of appetite. These changes were completely reversed by PGA. The anemia did not show a marked or consistent response to liver extract, but at times there was a partial response to this material which was suggestive of an interrelationship between PGA and the antipernicious anemia factor in the antagonist-induced deficiency in pigs. The crude antagonist was found by Goldsmith and co-workers[24] to prevent the metamorphosis of *Drosophila melanogaster*. This effect was partially overcome by adding PGA to the culture medium.

Franklin and co-workers[25] fed "x-methyl" PGA to dogs on a purified diet and produced a syndrome characterized by a loss of weight, a mild macrocytic anemia and leukopenia, and, in the terminal state, severe inanition and dehydration with alopecia, achromotrichia, pruritis, dermatitis, and ulcerations of the skin. The development of the syndrome was prevented by the addition of PGA to the diet, but the administration of refined liver extract had very little effect.

Recently, Hultquist, Smith, Seeger, Cosulich, and Kuh have described the preparation and purification of three methylated derivatives of PGA, namely the 10-methyl,[17] 9-methyl, and 9,10-dimethyl[26] derivatives. The first of these compounds, 10-methyl PGA, was found to be a powerful PGA antagonist for *S. faecalis* R., but it had a weak folic acid-like effect in an experiment with chicks.

The second derivative, 9-methyl PGA, was found to have very little activity in the *S. faecalis* R. assay. At a level of 30 to 100 mg. per kilo of diet, however, it was found to produce deficiency signs in rats and chicks similar to those seen with "x-methyl" PGA. The syndrome was completely prevented by increasing the level of PGA in the diet. The biological behavior of the third compound in the series, 9,10-dimethyl PGA, was some-

what surprising, for it was found to have a high potency in the *S. faecalis* assay, yet it appeared to be less active than 9-methyl PGA in tests with rats and chicks.

The synthesis of a powerful antagonist of PGA was described by Seeger, Smith, and Hultquist[16] in November, 1947. This compound, 4-amino PGA, was found to displace more than its own weight of PGA in the *S. faecalis* R. test. Here, apparently, was a new type of folic acid antagonist, characterized by an unusually high potency. Studies with this compound, sometimes termed "Aminopterin," have extended into various biological fields and have given rise to a rapidly expanding literature in experimental biology. The compound has been found to produce the following effects: toxicity with characteristic lesions in mice, rats, monkeys, and guinea pigs, an in-

TABLE 1
CLASSES OF PTEROYLGLUTAMIC ACID ANALOGS

Class	*Type*	*Example*	*Reference*
1	Purine analog	2-amino purine	13
2	Pteridine compounds	2,4-diamino-6,7 diphenyl pteridine	14 15
3	PGA with different substituents on pteridine nucleus	4-amino PGA	16
	or on side chain	N^{10} methyl PGA	17
	or on both	4-amino-N^{10} methyl PGA	18
4	Pteroylamino acids other than PGA	pteroylaspartic acid	12 19
5	PGA with modifications of pteridine nucleus	quinoxaline-2-carboxyl-yl *p*-aminobenzoyl-glutamic acid	20
6	PGA analogs with carboxyl of paba replaced by sulfonyl	N(-4-(((2-benzimidazol) methyl)-amino)-sulfonyl) glutamic acid	21

hibition of the growth of Rous sarcoma in chicks, an arrest of the development of *Drosophila*, and an inhibition of the response of the oviducts of chicks and frogs to estrogens.[27–32]

In an examination of the pharmacological effects of 4-amino PGA, Philips and Thiersch have concluded[33] that the compound produces an absolute immediate deficiency of PGA. A similar viewpoint was expressed by Stickney *et al.*[34] We are in agreement with this, although the poisonous nature of 4-amino PGA has given rise to speculation as to whether the substance should be regarded as a cytotoxic agent rather than as a pteroylglutamic acid antagonist. Consideration, however, will show that these two viewpoints are not mutually exclusive. First, the compound must necessarily be regarded as a PGA antagonist from results[30] which showed that the inhibitory effect of 4-amino PGA on the growth of *S. faecalis* R. was reversed by PGA, by pteroic acid, and by pteroyl-gammaglutamyl-glutamic acid or pteroyl-gammaglutamyl-gammaglutamyl-glutamic acid. As the concentration of the antagonist was increased, larger and larger proportions of the metabolite were required to neutralize the inhibition. Similar results are shown in TABLE 2. The tendency towards irreversibility with increasing

concentration was found to be even more marked in animal nutrition than in bacterial nutrition. Experiments with mice[27] and with chicks and rats[30] showed that 4-amino PGA was reversible by PGA over only an extremely narrow range above the threshold of its activity. No indication of reversibility by concentrated liver extract was noted. For mice, activity first appeared at 0.3 parts per million of diet, and at 1 ppm. the action of the antagonist was not abolished by 100 ppm. of PGA. With chicks, activity was first noted at 1 ppm. of diet. A level of 4 ppm. was reversed by 24 ppm. of PGA, but 5 ppm. of antagonist was not reversed by 25 ppm. of PGA. With rats, slowing of growth was first observed at 0.25 ppm. of antagonist in the diet. With 1 ppm., survival was obtained with 40 ppm. of PGA, but growth was only half-normal.

Table 2

Some Derivatives of Pteroylglutamic Acid and Their Biological Effects

PGA derivative	*Inhibition* of* S. faecalis *R. at three PGA levels (in mug per cc. of culture medium)*			*Toxicity for animals, expressed as ppm. of purified PGA-deficient diet for LD_{100}*			
	10	*100*	*1000*	*Mice*	*Rats*	*Chicks*	*Reverse†*
Crude "x-methyl"	30	20	30	1000	1000	1000	+
9-methyl	300	400	400	30 to 1000			+
10-methyl	1	1	0.8			Pro‡	
9,10-dimethyl	3	2	2	100	30	30	
4-amino	6	3	2	1	1 to 3	3	—
4-amino-9-methyl	2	2	2		10		
4-amino-10-methyl	2	0.5	0.3	1	3	5	—
4-amino-9,10-dimethyl	0.4	0.2	0.2	3	3	3	—

* Inhibition ratio to PGA for half-maximum growth.
† Reversible by PGA over a wide range.
‡ Slight PGA-like effect.

Although it may appear that the preceding discussion has labored a minor point, studies in clinical medicine have drawn attention to its importance, for the narrowness of the spread between the effective and toxic dosage rates of the compound is a serious limitation. Second, the structure of the molecule of 4-amino PGA is so nearly identical with that of PGA that it seems almost inevitable that the physiological activity of the analog is due to an interference with the functioning of the naturally occurring substance, pteroylglutamic acid, which appears to be present in all living organisms. Finally, a sudden and extensive blocking of the essential enzyme systems controlled by PGA has not been excluded as a plausible explanation of the cytotoxic changes produced by 4-amino PGA.

These considerations, coupled with the clinical interest in 4-amino PGA in the experimental treatment of leukemia, made it of great interest to prepare and examine derivatives of the substance in an effort to widen the range of safe dosage. It was found that the toxicity of the 4-amino compound, as measured by its effects on mice or rats on a purified diet, could be modified by changing the molecule. (a) Replacing glutamic acid by aspartic acid[35]

raised the LD_{50} markedly; replacing glutamic acid by alanine[36] removed the activity completely. The first of these two compounds, 4-amino pteroylaspartic acid, has been used clinically under the pseudonym "Amino-An-Fol." (b) Methylation of the C-9[37] or the N-10[18] atom slightly reduced the toxicity.

The nutritional properties of 4-amino-N^{10}-methyl PGA were studied,[38] and are of interest in view of the clinical use of this compound under the name "A-Methopterin." It was found to be a highly potent antagonist for PGA in the assay with *S. faecalis* R. Its toxicity for mice and rats was almost as high as that of 4-amino PGA, but it was decidedly less potent than the latter compound for chicks (see TABLE 2).

Recently, another derivative, 4-amino-9,10-dimethyl PGA[26] has been examined and has been found to be toxic for rats and chicks at 3 ppm. of diet. Reversal of the toxicity in the experiment with chicks, but not in those with rats, was produced by adding PGA, 10 ppm. of diet.

Farber and co-workers[39] have described how the use of 4-amino PGA in the attempted treatment of leukemia followed from earlier observations that hypoplastic changes in the bone marrow, without alterations in the course of the disease, resulted from administering pteroylaspartic acid to a leukemic patient. These earlier observations led to the subsequent use of PGA antagonists which were more potent than pteroylaspartic acid. Temporary remissions, often accompanied by toxic side-reactions, were produced in 5 children with acute leukemia treated with 4-amino PGA, one milligram daily. Several other clinical groups have described varying results obtained by the use of 4-amino PGA in leukemia.[34,40–45]

Shive and co-workers[46] have reported that thymidine was able to reverse the inhibitory effects of "x-methyl" PGA for *Leuconostoc mesenteroides* 8293, while Prusoff, Teply, and King[47] reported that a partial deficiency of PGA in a medium otherwise favorable for rapid growth of *Lactobacillus casei* resulted in a reduction in the rate of formation of desoxyribonucleic acid. These various observations appear further to relate PGA with nucleic acid metabolism and to emphasize the probability of a role for this vitamin in cellular proliferation. The diversity in the biological properties of the various PGA antagonists leads to the hope that the use of these substances may prove fruitful in investigating the chemistry of growth.

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FOLIC ACID ANALOGS IN LOWER ANIMALS.* I. THE INSECTA: *DROSOPHILA MELANOGASTER*†

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Thus far, the papers have been concerned with bacteria, fungi, mammal, and bird. This leaves quite a lacuna in our phylogenetic series. In an attempt to fill a portion of this gap, a brief account of the response of an invertebrate, namely *Drosophila melanogaster*, the fruit fly, and a lower vertebrate, *Rana clamitans*, a frog, to folic acid antagonists will be presented.

With the appearance of data suggesting that folic acid was of importance in certain types of tissue growth, it became of interest to ascertain the relation which might exist between folic acid level and the incidence and growth of hereditary tumors in the fruit fly. The availability of folic acid antagonists‡ and of ebony[11], a strain of *Drosophila* bearing a hereditary benign melanotic tumor, enabled us to make an attempt in this direction.

Technique and Stock. Pearl's[1] synthetic medium, S-101, modified by changing the agar content from 2.2 to 2.5 per cent, served as the basic food. The medium was supplemented with various levels of an impure compound, x-methyl PGA, the so-called "crude" antagonist (Lederle N 67), and of aminopterin (4-aminopteroylglutamic acid), a folic acid analog. Both of these were added to the medium alone and in combination with varying quantities of pteroylglutamic acid. Adequate mixing was assured by placing in a Waring Blendor for a short interval. The food was poured into 1×4 inch vials to a depth of $\frac{7}{8}$ inch, and a drop of Fleischman's live yeast suspension was placed on the surface of the food in each vial. The cultures were allowed to stand for 24 hours for yeast growth. Fifteen newly-hatched larvae were placed in each vial and incubated at $25° \pm 0.05°C$. The cultures were examined under the binocular dissecting microscope at intervals during the larval and subsequent developmental periods.

Results. Before proceeding, it is important to emphasize that the antagonists had no apparent effect upon the incidence or growth of the melanotic tumor. The mortality effects were striking, however, and it soon became evident that *Drosophila* was ideally suited as a test organism for folic acid requirements.

In TABLE 1, the adult survival values on the various levels of the x-methyl folic (N 67 Lederle) can be seen. At concentrations above 0.25 per cent of antagonist, the survival was 0 per cent as compared with 73.9 per cent adult survival for the untreated controls. What was happening to these animals? At which point in their life history did the antagonist operate?

* This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

† An abstract embodying preliminary observations in this connection appeared in the Anatomical Record. 1948. **101**:93.

‡ The pteroylglutamic acid and the 4-aminopteroylglutamic acid were generously supplied by Dr. T. H. Jukes, the late Dr. A. L. Franklin, and the late Dr. Y. SubbaRow of Lederle Laboratories Division of American Cyanamid Co.

At the lowest level of x-methyl folic, 0.1 per cent, there was a high percentage of pupae, most of which completed metamorphosis but only 4.7 per cent of which emerged as adult flies. At 0.25 per cent of drug, the pupae picture was about the same, but emergence was rare. At 0.5 per cent of

TABLE 1

THE EFFECTS OF SEVERAL CONCENTRATIONS OF A FOLIC ACID ANTAGONIST, X-METHYL FOLIC (N 67, LEDERLE), UPON ADULT SURVIVAL VALUES IN EBONY[11]

Gm. x-methyl folic / 100 cc. medium	0.10	0.25	0.50	0.75	1.00	2.00	3.00	control
Adults	7	3	0	0	0	0	0	709
Larvae	150	570	570	210	1590	300	600	960
% Survival	4.7	0.53	0.0	0.0	0.0	0.0	0.0	73.9

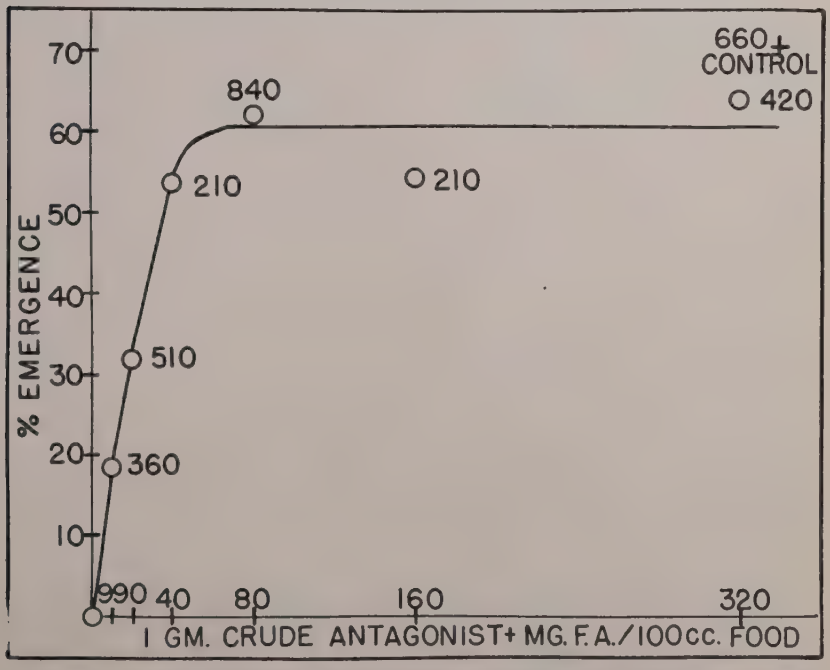

FIGURE 1. The effect of the addition of varying quantities of folic acid to the 1 per cent crude antagonist ration upon adult survival values in ebony[11]. The numbers next to the plotted points on the graph represent the number of larvae used.

the antagonist and higher, emergence became 0 per cent. There were, however, observable differences in the rates of development. At 1 per cent, the beginning of metamorphosis was retarded 24 hours. The larval period lasted five days instead of the typical four. Most of the animals survived half way through metamorphosis. At 2 per cent, there was a 36-hour delay in the onset of metamorphosis, with very few surviving to the half-way mark

to metamorphosis. At 3 per cent, the delay was 48 hours or 50 per cent. All animals died as prepupae or early in pupation (these flies just about reached the onset of metamorphosis and then died). To sum up, as the concentration of antagonist was increased above 1 per cent, fewer animals were able to begin their metamorphosis, let alone complete it. A number of these larvae lived for as long as 14 days, at which time they should have completed their metamorphosis and lived as sexually mature adults for about 6 days.

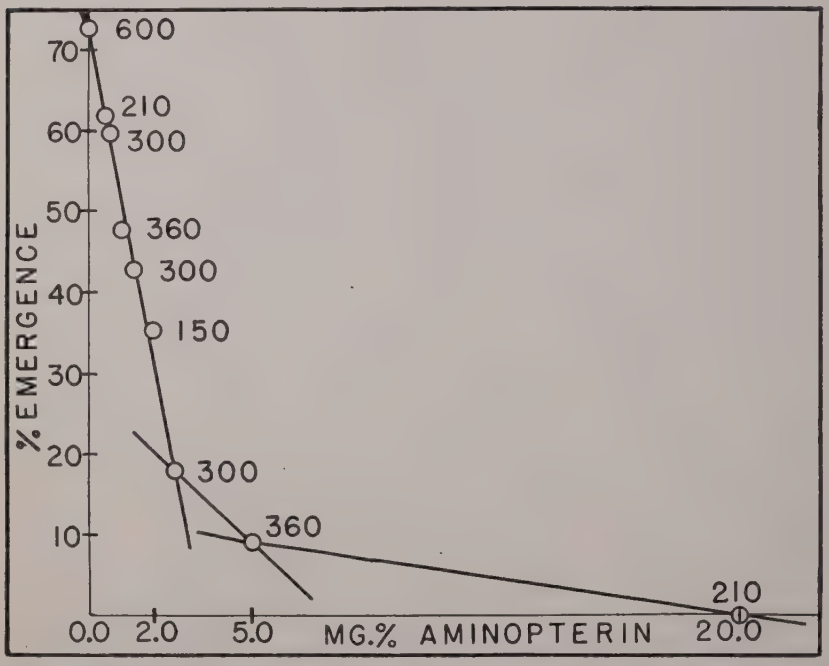

FIGURE 2. The effect of several concentrations of 4-aminopteroylglutamic acid upon the adult survival values in ebony[11]. The numbers next to the plotted points on the graph represent the number of larvae used.

To ascertain whether the antagonist effects of the x-methyl folic could be overcome with PGA, varying amounts of it were added to the medium. In FIGURE 1, the effects produced by supplementing the 1 per cent antagonist ration with several levels of PGA can be seen. As the concentration of folic acid was increased, there was a sharp rise in emergence values and, at 40 mg. of folic for each gm. of crude antagonist, 53 per cent survived, with the value proceeding asymptotically to about 63 per cent when 320 mg. of folic acid were added for each gram of antagonist. Seventy per cent of the untreated controls and none of the animals on the 1 per cent antagonist medium emerged. Runs supplemented with higher values of folic acid thus far have not yielded consistent data.

At the 0.5 per cent level of antagonist in the medium, it was found possible to overcome the antagonist completely with a dose of about 40 mg. of folic acid for each gram of x-methyl folic.

In FIGURE 2, per cent emergence is plotted against mg. per cent of aminopterin. The lowest concentration, 0.5 mg. per cent of aminopterin, yielded 61 per cent emergence. As the concentration was raised, the emergence value decreased at a steady rate until the 2.5 per cent level. Survival values continued to decrease, with 0 per cent emergence at 20 mg. per cent.* At the 5 mg. per cent concentration, where the emergence value was about 10 per cent, addition of folic acid in a 50 to 1 ratio appeared to double the number of emerged animals.† This compared with a survival value of about 72 per cent for untreated controls.

Discussion. It is apparent that incorporation of x-methyl folic acid or 4-aminopteroylglutamic acid in the basal food ration of *Drosophila* was followed by a retarded rate of development and in many cases failure to complete the life cycle. The antagonist effect could be annulled by the addition of suitable concentrations of PGA. It is of interest to note that it had been demonstrated[2] previously that folic acid is an important growth factor for several species of insects, and that the Aëdes mosquito requires folic acid for pupation.[3] Folic acid, however, is not the pupation hormone.[3] Previously, Fraenkel and Blewett[4] had concluded from a review of the literature that insects require essentially the same factors of the vitamin-B complex as do rats, chicks, and microorganisms.

In the light of these observations, a multicellular organism of known genetic stock is now available as a test animal for eliciting further basic information as to the functions of the newer members of the B complex and the nucleic acid fractions.

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* More recent results indicate that one begins to approach zero emergence at a level of 7.5 mg. per cent.

† Data accumulated since the conference indicate that supplementation with larger amounts of folic acid (100:1 at the 5.0 mg. per cent level) can increase the survival value to about 40 per cent for the ebony[11] genotype. The Woodbury (Wild-type) can be made to approach the control values by the addition of suitable quantities of folic acid. It is important to note that desoxyribonucleic acid (Schwarz), when used in low concentrations, has been found to be as active as PGA when used up to the limits of its solubility. Thymine is but slightly effective, and yeast nucleic acid is even less so.[5]

FOLIC ACID ANALOGS IN LOWER ANIMALS.* II. THE AMPHIBIA: *RANA CLAMITANS*

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It has been demonstrated previously that the oviducts of newly metamorphosed frogs show a marked response to injections of estradiol benzoate.[1] More recently, it has been reported that the genital tracts of female chicks and monkeys[2] receiving a diet deficient in pteroylglutamic acid (PGA) responded but slightly to the administration of estrogenic substances. A chemical antagonist of folic acid, x-methyl folic (crude antagonist, Lederle) when incorporated in the stock ration, yielded results similar to those obtained on a diet deficient in PGA.[3] PGA antagonists of known chemical composition such as Aminopterin (4-aminopteroylglutamic acid) and Amethopterin (4-amino-N^{10}-methyl pteroylglutamic acid) have been shown to inhibit the estrogen-induced tissue growth in the genital tract of the female chick[4,5] and mammal.[5]

In view of these observations, it was considered worthwhile to investigate the aminopterin-estrogen induced growth relationship in the frog.

Experimental. Several dosage levels of 4-amino PGA,† PGA,† and estradiol,‡ alone and in combination, were administered parenterally to newly metamorphosed frogs (*Rana clamitans*). In a second series, the frogs were pretreated with aminopterin alone, folic acid alone, or both for a period of 2 to 3 weeks. At the end of this period, estrogens were also given. The 4-amino PGA in dosages of 0.05, 0.10, 0.20, and 0.25 mg. was injected three times a week and in a dose of 1.0 mg. twice a week. PGA in amounts up to 5.0 mg. was injected three times a week. One tenth of one mg. of estradiol benzoate was given once a week. All precautions were taken to prevent post-injectional leakage of administered fluid.

For histological study, the animals were fixed in Bouin's, and serial sections of the craniad portion of the oviduct extending half or two-thirds of the kidney were cut at 7μ. Masson's, hematoxylin and eosin, and iron hematoxylin were used as stains. Comparable sections from each slide were examined, so that comparable areas in all the animals were studied to obtain estimates of mitotic activity throughout the length of the oviduct.

Observations. Macroscopically, the oviducts of the estrogen control animals were greatly enlarged and showed pronounced coiling. The growth response and the coiling elicited by the estradiol were enhanced by pre- and concomitant treatment with folic acid. Omission of the pretreatment with PGA resulted in oviducts whose appearance was similar to that of the frogs receiving only the sex hormone. In those animals in which aminop-

* This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

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† The pteroylglutamic acid and the 4-aminopteroylglutamic acid were generously supplied by Dr. T. H. Jukes, the late Dr. A. L. Franklin, and the late Dr. Y. SubbaRow of Lederle Laboratories Division of American Cyanamid Co.

‡ The estradiol benzoate was generously supplied by Dr. F. F. Yonkman and Dr. F. L. Mohr of Ciba Pharmaceutical Products.

terin and the estrogen were injected together, and in those which received the aminopterin for 2–3 weeks prior to the initiation of the estrogen regimen, the oviducts were but slightly enlarged and no coiling was discernible.

Microscopically, the small and straight oviducts in the untreated controls consisted of a single layer of cuboidal epithelium encircling the small lumen. Nuclei were regular oval or round. In the estradiol treated controls, the major portion of the oviducts was comprised of glands. These radiated from the lumina to the periphery of the oviducts. Granular cytoplasm and basal nuclei characterized the gland cells. Well-differentiated, ciliated, columnar epithelial cells containing regular oval vesicular nuclei lined the lumina.

In the oviducts of the aminopterin-estrogen treated animals, no evidence of gland formation was visible. The lumina were lined by a single layer of low columnar epithelium only slightly more prominent than that found in the untreated controls. Areas of cystic dilatation were observed, and in these regions the lumina were lined by flat epithelium almost squamous in appearance.

Addition of folic acid to the aminopterin-estrogen regimen was followed by a slightly increased infolding of the oviduct epithelium into the lumina. No other significant histological changes could be observed.

The mitotic counts of the oviduct epithelium revealed an interesting and puzzling phenomenon. In the untreated controls, estrogen controls, and estrogen-PGA group, mitotic figures were rare. In the first group, there was less than one mitotic figure per thousand cells; in the others there was less than one mitotic figure in 1,500 cells. In contrast to this picture, those animals which had received both estradiol and aminopterin numbered 12 to 36 mitotic figures per thousand cells. Metaphases were in the majority; many in other phases were also present.

In an attempt to overcome the "anti-folic" effect, folic acid in dosages of 10, 50, and 100 to 1 of folic acid to aminopterin were administered to the estradiol-aminopterin treated animals. The lower levels of PGA produced no apparent microscopic effect. The oviducts in the frogs on the highest concentrations appeared slightly larger when viewed with the aid of the binocular dissecting microscope. In the light of our more recent work and that of Hertz, it is likely that pretreatment with and the use of larger amounts of PGA might have served to negate the "anti-folic" action.

Discussion. It is clear that the growth and differentiation of the female genital tract which usually follow the administraion of estrogen did not obtain when an analog of pteroylglutamic acid, 4-aminopteroylglutamic acid, was injected concurrently. The oviducts of aminopterin-estrogen treated frogs, which differed but slightly in size from those in the untreated controls, contained many cells undergoing mitosis, whereas oviducts in other groups were almost completely devoid of such cells.* This apparent contradiction of a high mitotic count associated with little growth or differentiation may provide a clue and partial answer to Dr. Paschkis' query, thus

* It must be emphasized that, had the frogs which received estradiol alone been sacrificed at an earlier date, numerous mitotic figures would have been present in the oviduct epithelium. At the time of sacrifice, however, 2 to 3 weeks after the initiation of treatment, the period of active cell division was over.

far unanswered, as to the mechanism of action of the folic acid antagonists. To this end, the findings of cytologists, cytochemists, and biochemists are of special interest. The amount of available nucleic acid appears to be one of the chief factors which determines the rate of cell division.[6–8] King and his colleagues[9] have briefly summarized the experimental work pertaining to the functional relationship between pteroylglutamic acid and the synthesis of certain constituents of nucleic acids. Their own work revealed that *Lactobacillus casei* grown in a medium partially deficient in PGA exhibited a marked decrease in desoxyribonucleic acid. The content of ribonucleic acid remained unchanged. It is the former, desoxyribonucleic acid,† which is recognized as a universal constituent of cell nuclei.[10]

It is beyond the scope of this paper to survey the entire field, but we should like to draw attention to the following possibilities. A folic acid antagonist, such as 4-aminopteroylglutamic acid, by interfering with the utilization of folic acid may reduce nucleic acid synthesis and thus cause a marked retardation in the rate of cell divison.

Should these assumptions prove to be valid, they might serve to explain the growth inhibition which has been obtained with the folic acid analogs in forms as diverse as the bacteria, the insect, and the mammal in certain of the leukemias, and in the transplantable mouse Sarcoma 180.

Summary. Newly metamorphosed frogs (*Rana clamitans*) were treated parenterally with several dosage levels of 4-aminopteroylglutamic acid (aminopterin), pteroylglutamic acid (folic acid), and estradiol benzoate, alone and in combination. Aminopterin decreased, whereas folic acid increased, the growth response of the oviducts to estradiol. The aminopterin effect could not be reversed by folic acid in ratios as high as 100:1 of folic acid to aminopterin. Pretreatment with folic acid and the use of higher levels may have served to negate the antagonist effect.

A significant increase in the number of mitotic figures was observed in the epithelium of the oviducts of the estradiol-aminopterin treated frogs. An interpretation of the relation of these observations to the possible mechanism of action of the folic acid antagonists has been presented.

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† Recent experiments with *Drosophila melanogaster* in our laboratories (Goldsmith and Harnly)[11] have clearly demonstrated that low levels of desoxyribonucleic acid are as active as much larger doses of folic acid in counteracting the aminopterin action upon development and survival values in the fruit fly. Thymine is not as effective as folic acid, and yeast nucleic acid is but slightly effective.

STUDIES OF THE ACTION OF 4-AMINOPTEROYLGLUTAMIC ACID AND ITS CONGENERS IN MAMMALS*

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Evidence that synthetic analogs of folic acid (PGA) could act as antagonists of the vitamin in mammals was first obtained by Franklin and coworkers employing a crude preparation of an "x-methyl-folic acid" in rats and mice.[1, 2] These studies were later confirmed in pigs and mice.[3–5] When "x-methyl-folic acid" was added as a supplement to deficient diets in concentrations several thousandfold greater than that of PGA, characteristic signs of PGA-deficiency were accelerated in appearance and enhanced in severity. The syndrome was readily prevented or successfully treated by administration of elevated levels of PGA. Subsequent to the initial studies with "x-methyl-folic acid," a new series of antagonists was introduced with the synthesis of 4-amino-pteroylglutamic acid (4-amino-PGA).[6] Observations of the effects of this agent given as a dietary supplement to mice[7] and rats[8] proved it active in concentrations which were approximately equivalent to the amounts of PGA present in the diets administered. This finding is to be contrasted with the relatively low activity of "x-methyl-folic acid." Effective doses of 4-amino-PGA proved fatal within a few days after dietary supplementation. Furthermore, PGA protected against intoxication only when fed in high levels to animals ingesting minimally fatal concentrations of 4-amino-PGA.

In view of the high potency of 4-amino-PGA and its structural relationship to PGA, it seemed important to explore the nature of the lesions produced by the agent in order to obtain information concerning its mechanism of action. Such understanding was further necessitated by current interest in the compound and its congeners as substances having potential therapeutic value in certain neoplastic diseases.[9, 10, 10a] Accordingly, the present report is a brief summary of the actions of 4-amino-PGA, 4-amino-N^{10}-methyl-PGA, and 4-amino-pteroylaspartic acid (4-amino-PAA) in mice, rats, and dogs.[11–13]

Toxicity. Of initial interest to analysis of the mechanism of action was a study of the toxicity of the 4-amino analogs of PGA in the three species used. Animals receiving single, fatal doses survived for at least 48 hours and usually succumbed between the third and fifth day after poisoning. Only a few exceptional mice succumbed earlier than 48 hours when given doses as great as 1000 mg./kg. of 4-amino-PAA (TABLE 1). The toxicity of

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† Neale Research Pathologist, University of Adelaide, S. Australia; National Cancer Institute Fellow, U. S. Public Health Service.

‡ The authors are indebted to Drs. T. H. Jukes, the late Y. SubbaRow, and J. H. Williams of the Lederle Laboratories and to Drs. R. P. Parker and J. M. Smith, Jr., of the Calco Chemical Division, American Cyanamid Company, as well as their coworkers, for generous supplies and information concerning the purity and actions of the agents used in the present study.

TABLE 1

TOXICITY BY INTRAPERITONEAL ADMINISTRATION OF 4-AMINO-PGA, 4-AMINO-PAA, AND PGA IN MICE AND RATS*

Agent	*Species*	*Dose*	*Number of injections*	*Mortality*	*Day of death*						
					1	*2*	*3*	*4*	*5–7*	*8–10*	*11–14*
		mg./kg./day									
4-amino-PGA	mice†	10	1	23/24			11	11	1		
		5	1	16/24			7	5	3	1	
		2.5	1	8/24			2	5	1		
		1.25	1	0/24							
		0.63	1	1/24				1			
		0.8	5	12/12			4	2	6		
		0.4	5	12/12				3	8		1
		0.2	5	2/12					2		
		0.1	5	0/12							
	rats‡	40	1	12/12			3	6	3		
		20	1	11/12			5	3	3		
		10	1	22/24			2	10	9		1
		5	1	18/23		1	3	3	8	1	2
		2.5	1	4/17				1	1		2
		1.25	1	3/18					1	1	1
		1.0	5	6/6				5	1		
		0.5	5	6/6			1	3	2		
		0.25	5	5/6				1	3	1	
		0.125	5	2/6						1	1
4-amino-PAA	mice†	1000	1	12/12	2	8		1	1		
		500	1	24/24		10	7	6	1		
		250	1	15/24			3	3	5	4	
		125	1	1/24					1		
		250	5	12/12		1	3	8			
		125	5	24/24			4	6	14		
		62.5	5	15/24				5	9	1	
		31.3	5	1/24					1		
	rats‡	1000	1	11/12			4		7		
		500	1	11/12				2	8		1
		250	1	7/12				2	2		3
		125	1	0/12							
		250	5	12/12			1	7	4		
		125	5	12/12				1	10	1	
		62.5	5	7/12					3	4	
		31.3	5	2/12							2
PGA	mice†	1000	1	23/24	22		1				
		500	1	24/24	2	7	11	4			
		250	1	20/24	1	3	14	2			
		125	1	2/24			1	1			
		250	5	12/12		4	5	2	1		
		125	5	6/12			1	1	3		1
		62.5	5	0/12							

* Data for mice not previously reported. Data for rats include results of previous tests.[11]
† AKM strain, 17.5 to 22.5 grams, equal numbers of both sexes used at each dose.
‡ Wistar albino, 100 to 200 grams, approximately equal numbers of both sexes tested at each dose.

TABLE 2

MEDIAN LETHAL DOSE, LD_{50}, IN MG./KG. OF 4-AMINO CONGENERS OF PGA IN MICE, RATS, AND DOGS*

Agent	*Mice*				*Rats*				*Dogs*
	acute		*chronic*†		*acute*		*chronic*†		*chronic*‡
	LD_{50}	*S*	LD_{50}	*S*	LD_{50}	*S*	LD_{50}	*S*	
4-Amino-PGA	3.6 (2.5–5.2)	2.4 (1.7–3.5)	0.24 (0.20–0.28)	1.3 (1.2–1.4)	3.4 (2.4–4.9)	2.7 (1.9–3.9)	0.15 (0.10–0.23)	1.6 (1.1–2.4)	0.05–0.10
4-amino-N^{10} methyl-PGA[12]	89 (72–110)	2.4 (2.0–2.8)	2.0 (1.4–2.8)	1.9 (1.4–2.4)	14.7 (7.7–28)	7.2 (2.5–20)	1.2 (0.6–2.3)	2.6 (1.3–5.5)	0.2—1.0
4-amino-PAA	225 (180–270)	1.4 (1.2–1.6)	56 (46–68)	1.4 (1.2–1.6)	270 (180–400)	2.0 (1.4–2.9)	55 (40–76)	1.8 (1.2–2.5)	1.0–5.0
PGA	190 (160–220)	1.3 (1.2–1.4)	125 (110–140)	1.2 (1.1–1.2)					

* Statistics calculated by the methods of Wilcoxon and Litchfield.[17] Confidence limits for 19/20 probability are included in parentheses under values for LD_{50} and S, slope function.

† Daily dose, 5 successive days.

‡ Approximate daily dose fatal in 10 days to 50 per cent.

4-amino-PGA and 4-amino-N^{10}-methyl-PGA was increased in mice and rats by administration in multiple doses as compared with the activity of single doses. While tests with 4-amino-PAA failed to indicate an enhancement of toxicity by chronic administration, the agent proved equally active following single or multiple injections. Such results are to be contrasted with the more usual finding with mice and rats that most substances can be administered daily in large fractions of their lethal dose without fatal consequences. As an example of the typical relationship between acute and chronic toxicity were observations in mice given PGA (TABLES 1, 2, and 3). Another characteristic feature of the actions of the 4-amino congeners of PGA was the greater variability found in toxic responses to administration of single doses than to multiple doses. The less precise relationship between mortality and dosage noted in acute poisoning is apparent statistically in the values for S (slope function) shown in TABLE 2.

TABLE 3

A COMPARISON OF ACUTE WITH CHRONIC TOXICITY OF 4-AMINO CONGENERS OF PGA IN MICE AND RATS

	Acute LD_{50}/Chronic LD_{50} × 5	
	mice	*rats*
4-amino-PGA	3.0*	5.2*
4-amino-N^{10}-methyl-PGA	9.0*	2.35†
4-amino-PAA	0.8†	1.0†
PGA	0.3‡	

* Significantly greater than 1.0 for $P \leqq 0.05$.
† Not significantly different than 1.0 for $P \leqq 0.05$.
‡ Significantly less than 1.0 for $P \leqq 0.05$.

In view of this finding it seemed reasonable to limit comparisons of the toxicity of the agents in the different species tested to the results of chronic administration. Such comparisons revealed the following (TABLE 4): (1) in each species, 4-amino-PGA was most toxic and 4-amino-PAA least toxic; (2) to each of the agents, rats were somewhat more sensitive than mice, while dogs were considerably less tolerant than either of the rodents; and (3) the toxicity of 4-amino-PAA compared with that of the other congeners differed less in dogs than in mice or rats.

In view of the widely differing potencies of the 4-amino congeners of PGA in mammals, it is important to inquire whether variations in activity in higher organisms are to be related to differences in cellular susceptibility or to relative differences in distribution between tissues of varying susceptibility as well as differences in the extent to which each of the agents is excreted or converted by metabolism into inactive products. Since, in the microorganism, *Streptococcus faecalis* R, the agents are approximately of equal potency as antagonists of PGA,[14,15] it is not unreasonable to propose that differences in cellular susceptibility are less significant than other pharmacodynamic factors in accounting for differences in activity in mammals.

Course of Intoxication. Manifestations of acute, fatal intoxication with the 4-amino congeners of folic acid in mice, rats, and dogs included progressive weight loss, anorexia, diarrhea, which was initially yellowish-brown in color and eventually often grossly stained with blood, progressive de-

TABLE 4

RELATIVE VALUES FOR LD_{50} OF 4-AMINO CONGENERS OF PGA IN MICE, RATS, AND DOGS

	Mice	*Rats*	*Dogs*
4-amino-PGA	1	1	1
4-amino-N^{10}-methyl-PGA	6–12*	4–17*	5–20
4-amino-PAA	180–310*	210–610*	10–100
PGA	410–660*		

* Confidence limits for 19/20 probability.

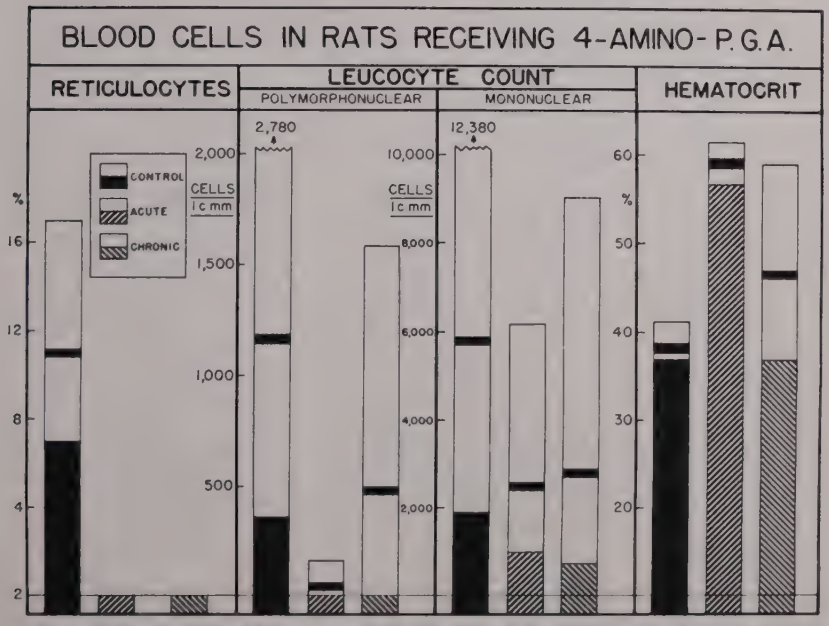

FIGURE 1. Clear zone in each bar represents range of maximum and minimum values. Lines crossing clear zones are average values. "Acute" refers to values 72 hours after administration of 40 mg./kg., intraperitoneally. "Chronic" refers to values after administration of 36 to 37 doses which were initially 0.05 and later 0.1 and 0.2 mg./kg./day during a period of 54 to 55 days.[11]

pression, and terminal collapse and coma. While losses in body weight were observed within 24 hours after intoxication, diarrhea appeared only after 48 hours. In animals receiving the agents by repeated administration, similar morbid changes appeared in the same order but with time of onset delayed.

Pathological Changes in Rats. The marrow of the femur of rats receiving large, single injections of 4-amino-PGA (40 mg./kg., about $10 \times LD_{50}$) was

converted from a grayish-red, gelatinous material to a darker, more fluid substance by the twelfth hour after intoxication. Progressive liquefaction of the marrow occurred until, at 72 hours, only purple fluid could be found. In the peripheral blood, marked granulocytopenia, reticulocytopenia, and moderate lymphopenia developed simultaneously. FIGURES 1 and 2 illustrate the extent of these changes in blood and bone marrow. It is important to note that significant depletion of nucleated erythroid elements in bone marrow was evident as early as 24 hours after acute poisoning, while depression of myeloid elements was found at 48 hours. The elevated proportions of lymphoid cells in marrow were due to replacement of marrow

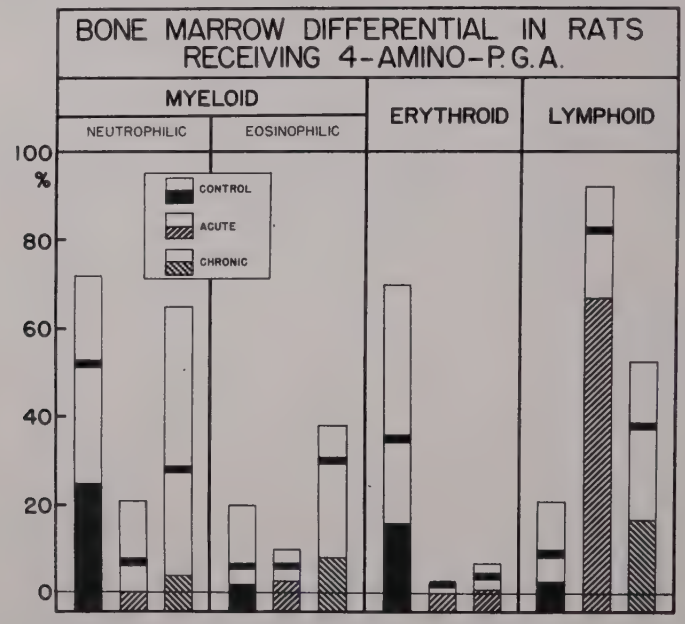

FIGURE 2. Values taken from same animals described in FIGURE 1. "Erythroid" refers to nucleated erythroid cells.[11]

tissues by blood. Similar changes in both blood and bone marrow were observed in rats poisoned by chronic administration of 4-amino-PGA as depicted in FIGURES 1 and 2.

In microscopic sections prepared for histological study of sternal and femoral marrow of rats, ill-defined areas of degeneration were observed as early as 6 to 12 hours after administration of 40 mg./kg. of 4-amino-PGA. After 24 and 48 hours, the sinus and capillary network of the marrow became prominent due to depletion of erythro- and myelopoiesis. Between 48 and 72 hours, hematopoietic tissues largely vanished from the marrow, leaving a network of capillaries and sinusoids filled with blood, with only eosinophils, megakaryocytes, and a few basophil normoblasts and erythroblasts remaining.

The rapid and extreme response of the bone marrow of the rat has been used to advantage by Ingle, Thiersch, and Karnofsky (unpublished observations) in testing the possible role of visceral organs in the mechanism of toxic action of 4-amino-PGA. Since typical damage could be elicited in eviscerated rats, it would appear that the effect of 4-amino-PGA on cells of the bone marrow is a direct one.

The lesions of erythropoietic and myelopoietic tissues just described in rats following injection of 4-amino-PGA were found to be similar in nature and severity after administration of toxic doses of 4-amino-N^{10}-methyl-PGA and 4-amino-PAA. Similarly, the adverse actions of 4-amino-PGA on the intestinal tract of rats were shared by the other 4-amino folic acids studied. Thus, at time of sacrifice, all animals had a natural oral mucosa and esophagus. However, the stomach and intestinal canal were filled in most cases with a yellow-brownish fluid and were often distended. Parts of the colon and rectum were spastically contracted.

Microscopic examination of tissues from the small and large intestine as early as 6 and 12 hours after administration of 40 mg./kg. of 4-amino-PGA revealed swelling and cytoplasmic vacuolation in the epithelial lining. Distal portions of villi were distended by capillary and venule dilatation. The initial changes were followed progressively by desquamation of epithelium, plasma extravasation into the intestinal lumen, and intensive leucocytic infiltration of the distended and denuded villi. At 72 hours after acute poisoning, the whole mucous membrane from duodenum to anus and especially in the colon was edematous, swollen, and, in part, hemorrhagic. Similar findings were obtained as the result of chronic intoxication.

Apart from the lesions of the bone marrow and the intestines, no outstanding, primary pathological changes were noted in rats. While lymph nodes and lymph follicles of the spleen, as well as lymphatic placques of the intestine, were decreased in size, and lymphopenia occurred as the result of poisoning, such lymphoid alterations were moderate and may be related in part to migration of lymphocytes to the wall of the gut, where they were found in abundance. In addition, a few animals, especially during chronic intoxication, developed salmonella infections which probably followed primary intestinal injury. The infections were ascending and caused enlargement of mesenteric lymph nodes, abscess formation, multiple fibrinoid necrosis in liver, spleen, kidney, and lung, bronchitis, and bronchopneumonia.

At this point, it is fruitful to note that the pathology of rats receiving 4-amino-PGA and its analogs resemble changes previously ascribed to effects of PGA deficiency in this species.[16] Pertinent in this regard is the hypocellularity of the bone marrow resulting from depletion of both myeloid and erythroid elements. In addition, lymphoid tissues are relatively spared and no other signs of primary damage to specific tissues are found.

Bone Marrow Changes in Mice. In mice treated with daily doses of either 4-amino-PGA or 4-amino-N^{10}-methyl-PGA, a lesion of the bone marrow developed[18] which resembled changes recently described by Weir, Heinle, and Welch in animals of the same species fed PGA-deficient diets and x-methyl folic acid.[5] Thus, the marrow of all treated animals was cellular

but, at the same time, deficient in nucleated erythroid elements. Depletion of mature myeloid forms was proportionately less marked than in the erythroid series, with the exception of an occasional animal in which polymorphonuclear neutrophils were also reduced in number.

Pathological Changes in Dogs. The destructive actions of 4-amino-PGA and its congeners on the intestinal canal, as described in rats, were also prominent in dogs. At time of sacrifice, the intestinal canal of animals in terminal stages of intoxication contained a dark, blood-stained fluid. Hemorrhagic enteritis of the duodenum, ileum, and jejunum and desquamating colitis were present in all animals. The hemorrhagic diarrhea and consequent dehydration associated with the intestinal lesions were undoubtedly major factors in fatal intoxication. Diminution of plasma chloride levels and increase in volume of packed red cells in blood occurred in all dogs, while most animals evinced a simultaneous increase in plasma protein levels. Nonprotein nitrogen levels of plasma were not elevated, except in a few moribund animals. Alterations in blood glucose concentration were variable and failed to suggest primary changes in carbohydrate metabolism.

Significant derangements of hematopoiesis occurred during the course of acute and chronic intoxication in dogs. Studies of peripheral blood revealed consistent reticulocytopenia, granulocytopenia, and lymphopenia. In acutely intoxicated animals, serial aspirations revealed rapid degeneration of the bone marrow. Within 24 hours, disturbances in the maturation of erythrocytes could be found. These included disintegration of nuclei of normoblasts in mitosis, abnormal expulsion of nuclear fragments into the cytoplasm, and the development of increased numbers of cells containing nuclear remnants and Howell-Jolly bodies. Normal erythroid precursors, such as the basophilic normoblast and erythroblast, exhibited an alteration of nuclear pattern towards a fine chromatin network with development of parachromatin. Thus, within 24 hours, significant proportions of primitive erythroid elements were present as cells which could unequivocally be classified as megaloblasts. These appeared to undergo hemoglobinization, to exhibit disturbances in mitosis, and to give rise to cells with enlarged cytoplasm containing irregular-shaped nuclear remnants and Howell-Jolly bodies. In addition to basic alterations in the development of erythroid elements, proliferation of both normal and abnormal erythroid precursors was inhibited. Thus, within 72 to 96 hours after acute intoxication, marrow samples were found to be largely depleted of nucleated erythroid cells. Similar but less rapid degeneration followed chronic intoxication, although megaloblasts were not found in all animals so treated (FIGURE 3).

Rapid pathological alterations were also observed in myelopoiesis, with hypersegmentation of polymorphonuclear neutrophils and the appearance of giant metamyelocytes and myelocytes with disturbed nuclear-cytoplasmic relationships. Inhibition of proliferation was evident in primitive, myeloid cells, since in terminal stages of both acute and chronic intoxication only small numbers of myeloid elements could be found.

Examination of lymphatic tissues in nodes, spleen, thymus, and intestine

revealed diminution in content of lymphoid cells but no evidence for necrotic changes. It would appear that damage to lymphoid tissues in the dog, like the rat, was less severe than damage to myeloid or erythroid cells in bone marrow. An occasional dog developed pulmonary complications of infectious origin. No other evidence for primary pathological changes was observed.

The rapid induction of megaloblastic erythrogenesis in the dog, following administration of 4-amino-PGA and its analogs, is important evidence bear-

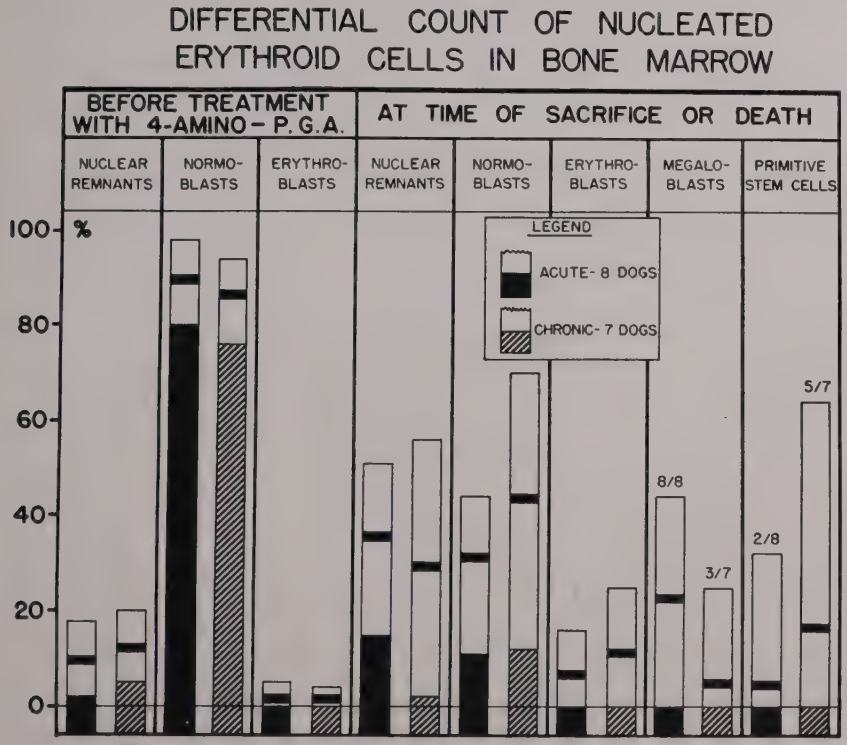

FIGURE 3. "Acute" refers to animals receiving .5 or more mg./kg., intravenously, in single doses. "Chronic" refers to repeated dosage of either 0.05 or 0.1 mg./kg./day. Fractions above last 4 bars indicate number in each group in which the designated cell types were found during serial studies of bone marrow.[13]

ing on the mechanism of action of these substances. The association of megaloblastic transformation in erythroid precusors with PGA deficiency is well substantiated in experimental studies with pigs[3,4] and in various human macrocytic anemias which respond to therapy with folic acid (see review of Jukes and Stokstad[19]). Moreover, in studies of patients undergoing therapeutic trials with 4-amino congeners of folic acid,[20] conversion of primitive erythroid precursors into megaloblastic forms has been observed with satisfying regularity in a manner resembling the similar changes noted in dogs.[21]

Conclusions

The observations just presented concerning the sites and characteristics of action of 4-amino-PGA and its analogs in mice, rats, and dogs as well as the reports of other workers using rats[8, 22] and guinea pigs,[23] lead to the conclusion that administration of the 4-amino congeners in effective doses induces signs of PGA deficiency. The extreme nature of the derangements noted and the rapidity of their onset warrant the use of the term "absolute deficiency" in descriptions of the syndrome reproduced by these agents. The fact that the actions of 4-amino-PGA are difficult to prevent in a reversible fashion by simultaneous administration of PGA remains unexplained, and its understanding may await definitive studies in isolated enzyme systems involving folic acid as a functional constituent. It is important, however, to consider certain properties of 4-amino-PGA which may bear on the irreversibility of its actions in mammals.

From the evidence just presented, the degenerative changes induced by the agent are rapid in appearance. Treatment of intoxicated animals with PGA must then be directed toward replacement of tissues which have become extensively necrotic and depleted.

Finally, under experimental conditions employing simultaneous administration of the metabolite and its potent analogs, transient changes in the relative proportions of the agents *in vivo* may at times reach balances unfavorable to the continued survival of cells dependent on folic acid. Such alterations in proportion of metabolite and its antagonists could be related to differences in their rates of excretion or metabolic conversion.

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FOLIC ACID ANALOGS AND EXPERIMENTAL TUMORS

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Earlier papers in this monograph have reviewed the classic studies on antimetabolites[1] and reported recent extensions[2] of this profitable concept. Approximately three years ago, the extensive, effective studies demonstrating the structure of folic acid and some of its biological activities were reported in a symposium of the Academy.[3] It was a natural consequence that analogs of folic acid should be prepared and studied for both folic and antifolic acid activity. Many of the subsequent findings in bacteria and animals have been reported in this monograph.[4–9]

In view of the essential role of folic acid in the growth of certain cells, it was logical that the folic acid antagonists be studied for adverse effects upon tumor tissue. It could have been predicted from the studies of folic acid deficiencies that compounds possessing antifolic acid activity would not be without adverse effects upon normal mammalian cells. Jukes *et al.*[6] and Philips *et al.*[9] have just reported studies indicating the nature of toxic effects of 4-amino pteroylglutamic acid and its congeners in mammals. It was with much of this background that studies on folic acid analogs were initiated in the hope that compounds would be found in which the toxicity to abnormal cells would be much greater than that for the most susceptible normal cells.

For the past year, the Division of Experimental Chemotherapy of the Sloan-Kettering Institute has concentrated study upon nearly 300 analogs of folic acid and simpler related compounds such as the pteridines, pyrimidines, and purines in a search for adverse effects upon abnormal tissue in a number of experimental conditions. This paper will be confined to studies of experimental animal tumors even though some of the compounds have reached clinical trial in a number of institutions.[10–18] The folic acid antagonists included in this discussion are 4-amino pteroylglutamic acid, 4-amino-N^{10}-methyl pteroylglutamic acid, 4-amino pteroylaspartic acid, and 2,6-diaminopurine (FIGURE 1).

Compounds have been received from a number of sources.† After the compounds are filed and coded for future reference, they may go directly for test against mouse tumors in tissue culture or in egg culture. Prior to the study against leukemia and Sarcoma 180 in mice, the compounds are submitted to the Pharmacology Section for determination of toxicity. Compounds of sufficient interest are studied in all of the tests and also submitted to trial against a spectrum of tumors in mice and rats. Compounds

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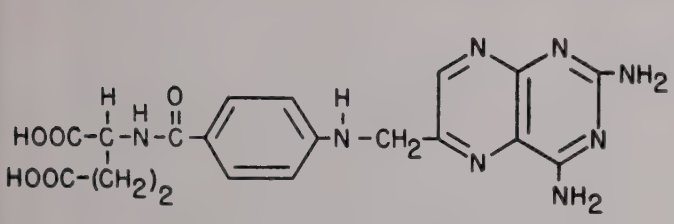
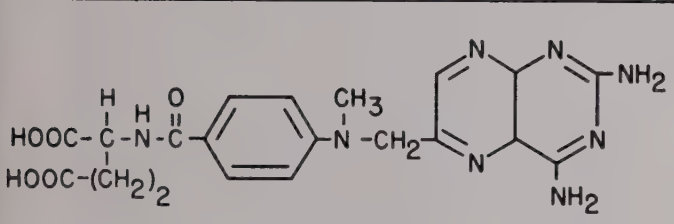
† We acknowledge our indebtedness to the Lederle Laboratories and Calco Divisions of the American Cyanamid Company for the folic acid analogs and to the Wellcome Research Laboratories for 2,6-diaminopurine.

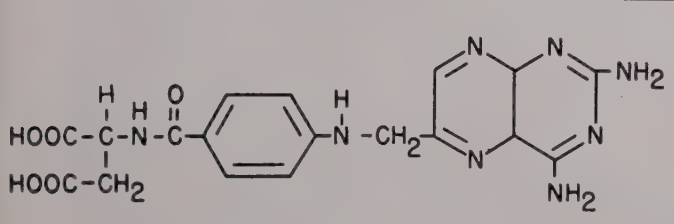
showing adequate activity against tumor tissue in one or more of the tests are considered for clinical trial. Before test of a substance in the clinic, more extensive studies of its pharmacology are conducted.[9]



4-AMINO-PGA



4-AMINO-N^{10}-METHYL-PGA



4-AMINO-PAA

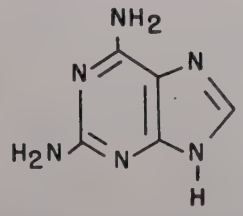


FIGURE 1. Formula of 4-amino-PGA(4-amino pteroylglutamic acid), 4-amino-N^{10}-methyl-PGA (4-amino-N^{10}-methyl pteroylglutamic acid), 4-amino-PAA (4-amino pteroylaspartic acid) and 2,6-diaminopurine.

Tissue Culture. Materials of particular interest or those available in limited amounts have been studied in tissue culture for differential toxicity to mouse tumor and embryonic tissue. Obviously, the information thus provided is characterized by the advantages and disadvantages of an *in vitro* test, wherein the modifying influence of the intact animal is eliminated.

The tissue culture studies have employed Gey's roller tube technique.[19, 20] Test tubes are made up holding rows of 6 explants of embryonic Akm mouse

skin and 2 similar rows of explants of sarcomas MA 387 and T 241 under a chicken plasma clot. The nutrient consists of 4 parts Gey's salt solution, 2 parts chick embryo extract, 1 part of human placental serum, and 3 parts of horse serum. The tubes are placed in a rotor for incubation 24 hours at 37°C., after which the cultures are examined for extent of growth and clot lysis and for damage as expressed largely in rounding up, granulation, and disintegration of the cells. The summation of the condition of the cells serves as a base line for damage suffered during exposure for the following 24 hours to compounds under study. The required concentration of test material, predetermined in a similar test on normal cultures, is added to the nutrient for exposure to the cultures. Duplicate or triplicate tubes are used with the maximum dose tolerated by normal tissue as well as with higher and lower concentrations. The two tumors used have shown different susceptibilities to a number of agents and thus indicate the desirability of using more than one tumor in such studies.

TABLE 1
FOLIC ACID ANTAGONISTS IN TISSUE CULTURE STUDIES

Compound	*Millimolar concentrations at approximate damage threshold for tissues*		
	Embryonic	*MA 387*	*T 241*
Folic acid	6.8*	6.8*	0.23
Teropterin	4.4*	4.4*	4.4*
4-NH_2-PGA†	5.8‡	2.9	2.9
4-NH_2-N^{10}-Me PGA	5.8*	5.8*	3.9
4-NH_2-PAA†	5.4	5.4	4.7
2,6-diamino purine	0.54	0.27	0.27

* No damage at highest concentration tested.
† PGA = pteroyloglutamic acid; PAA = pteroylaspartic acid.
‡ Solubility limitation.

The results obtained in studies with folic acid, a few of its analogs, and 2,6-diaminopurine are presented in TABLE 1. Though preliminary, the data indicate the concentrations of these agents which represent the approximate damage thresholds. It is of interest to recall that colchicine is capable of giving selective damage at a concentration of approximately .001 millimolar. The only striking differential toxicity is exhibited by T 241 with respect to folic acid. However, treatment of mice bearing Sarcoma T 241 with 100 mg./kg./day of folic acid has not inhibited development of the tumor.[21] 2,6-Diaminopurine was tested and found to be tolerated by normal tissue at twice the concentration safe for sarcoma tissue. FIGURES 2 and 3 reveal the effects observed with normal and T 241 cells exposed to 75 γ/cc. (0.41 mM) of 2,6-diaminopurine. It is to be noted that the treated normal cells retain their elongate spindle shape and appear undamaged, while, with T 241, a considerable increase in rounding of cells is observed together with some cellular disintegration. A combination of an antifolic compound with 2,6-diaminopurine appeared to increase the intensity of damage to the malignant cells.*

* Suggested by Dr. George Brown, based on a report by Hitchings.[22]

Egg Culture. Another method for study of substances against tumor tissue utilizes the chick embryo. Mouse and some human tumors can be grown upon the chorioallantoic membrane of the developing chick em-

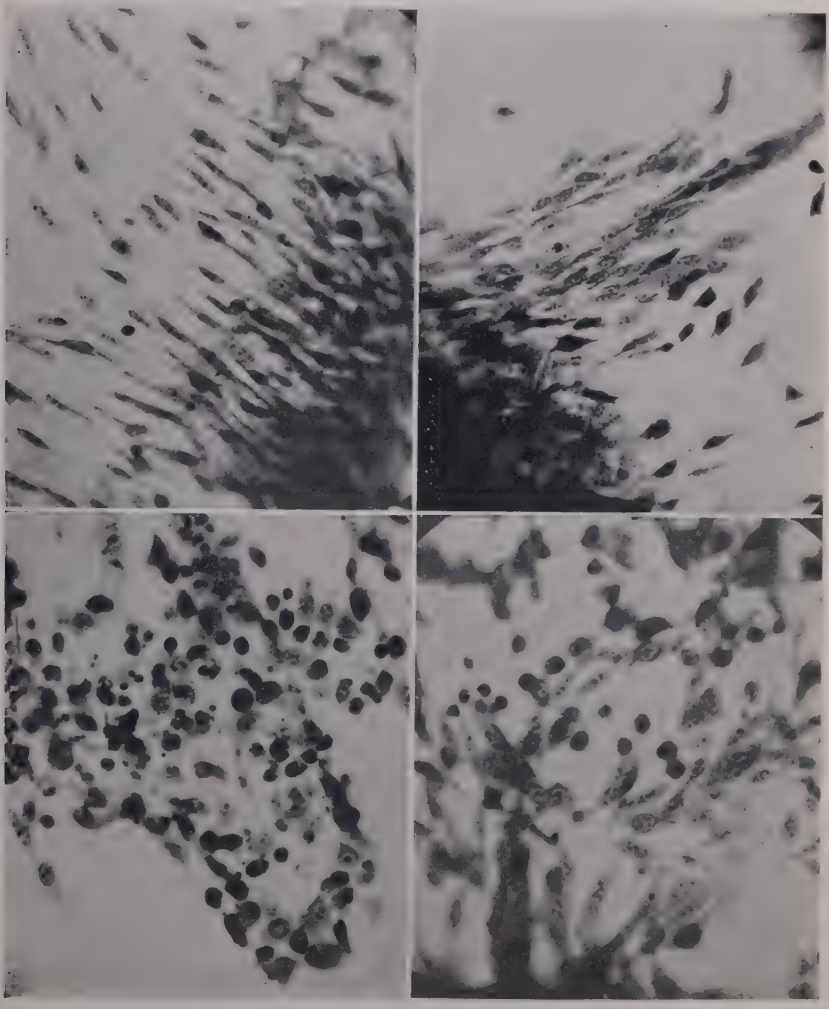


FIGURE 2 (*above*). Akm mouse skin in tissue culture. Right, untreated tissue control. Left, tissue exposed to 2,6-diaminopurine, 0.41 mM for 24 hrs. Note lack of damage.

FIGURE 3 (*below*). Mouse sarcoma T241 in tissue culture. Right, T241 untreated control. Left, T241 exposed to 2,6 diaminopurine, 0.41 mM for 24 hrs. Note damage of treated cells as indicated by increased rounding and some cellular disintegration.

bryo.[23] In this technique, fragments of mouse tumors, such as Sarcoma 180, are placed upon the membrane of an 8-day-old fertile chicken egg and, after four days' growth of the tumor, the maximum dose of chemical nonlethal to the chick is injected into the yolk sac. Five days later, the egg is

opened and the tumor removed for histological study and bioassay in mice. In this manner, it is possible to show extensive damage to Sarcoma 180 from use of nitrogen mustards at doses tolerated by the embryo.[24]

The toxicity of methyl bis (beta-chloroethyl) amine hydrochloride and X rays to the 12-day chick embryo and the effects of these agents on the Sarcoma 180 and Ma387 are shown in TABLE 2. The Sarcoma 180 grows readily in the egg to form cuboidal cells of relatively uniform size, and numerous mitotic figures are seen. The growing cells acquire an excellent blood supply (FIGURE 4). The yolk sac injection of 0.05 to 0.10 mg. per egg of a nitrogen mustard (methyl bis(beta-chloroethyl) amine hydrochloride), a dose which is not lethal to the embryo, induces severe injury to the Sarcoma 180 (FIGURE 4). Three to four days after the injection, many of the cells have disintegrated and disappeared, and the damaged area is invaded by chick leucocytes. Scattered, enlarged cells, with enormous

TABLE 2

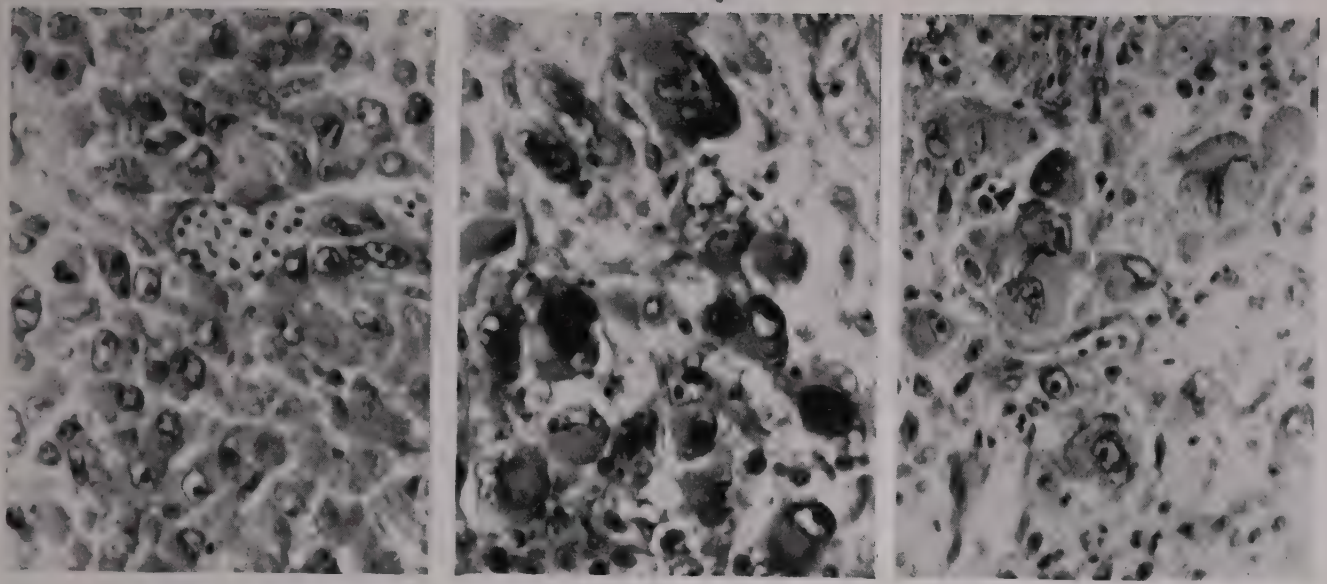
EFFECT OF NITROGEN MUSTARD AND OF X RAYS ON SA180 AND MA387 GROWING ON THE CHORIOALLANTOIC MEMBRANE

Effect	*Methyl-bis (β-chloroethyl) amine hydrochloride*	*X rays (6 r/minute)*
LD_{50}, 12 day embryo	0.3 mg./egg	1000–1200 r.
Chemotherapeutic dosage	0.05–0.10 mg./egg	1200 r.
Effect on Sarcoma 180		
Histological	severe damage	severe damage
Bioassay	no growth	no growth
Effect on Ma387		
Histological	moderate damage	moderate damage
Bioassay	decreased growth	decreased growth

and deformed nuclei, remain, and these cells are no longer viable when transp'anted to mice. A very similar effect on the Sarcoma 180 is produced by 1,200 r. of X rays to the egg.

Ma387, a tumor from the Akm strain of mice, forms bundles of spindle cells in the egg, growing in different directions. This tumor grows as fast as the Sarcoma 180, and it acquires an excellent blood supply. These cells are more resistant than the Sarcoma 180 to nitrogen mustard and X rays. Three to 4 days after exposure to nitrogen mustard, the cells are enlarged and show considerable variation in size. The nuclei are often distorted and mitotic figures are rare and usually abnormal. It is likely that many of these cells will ultimately undergo degenerative changes, although some of the cells are still viable when transplanted to mice after they received a dose of nitrogen mustard lethal to the Sarcoma 180. An exposure to 1,500 r. of X rays has only a slight histological effect on the Ma387.

TABLE 3 shows the toxicity and chemotherapeutic effects of folic acid and the well-known "antifolic" compounds in 12-day embryos and their effect on the Sarcoma 180 and Ma387. The Sarcoma 180 shows an interesting histological alteration. The cells become intensely vacuolated and then disintegrate (FIGURE 5). A few healthy-looking cells remain, and they are



CONTROL

AFTER NITROGEN MUSTARD
0.1 mg./egg

AFTER X RAYS

FIGURE 4. Sections of mouse sarcoma 180 growing on the chorioallantoic membrane of the chick embryo. Note blood supply in control tissue and in the treated sections, the enlarged cells with enormous deformed nuclei and numerous disintegrated cells.

TABLE 3

EFFECT OF TEROPTERIN AND THE "ANTIFOLIC" ACIDS ON SA180 AND MA387 GROWING ON THE CHORIOALLANTOIC MEMBRANE

Effect	*Pteroyl Tri GA*	*4-Amino PGA*	*4-amino N^{10}-methyl PGA*	*4-Amino PAA*
LD_{50}, 12 day embryo	>20 mg. egg	0.01 mg. egg	0.03 mg. egg	1–2 mg. egg
Chemotherapeutic dosage	0.005–20.0 mg.	0.05–0.10 mg.	0.1–0.2 mg.	2 mg.
Effect on Sarcoma 180				
Histological	no effect	severe damage	severe damage	severe damage
Bioassay	growth	growth	growth	growth
Effect on Ma387				
Histological	—	moderate damage	—	—
Bioassay	—	no effect	—	—

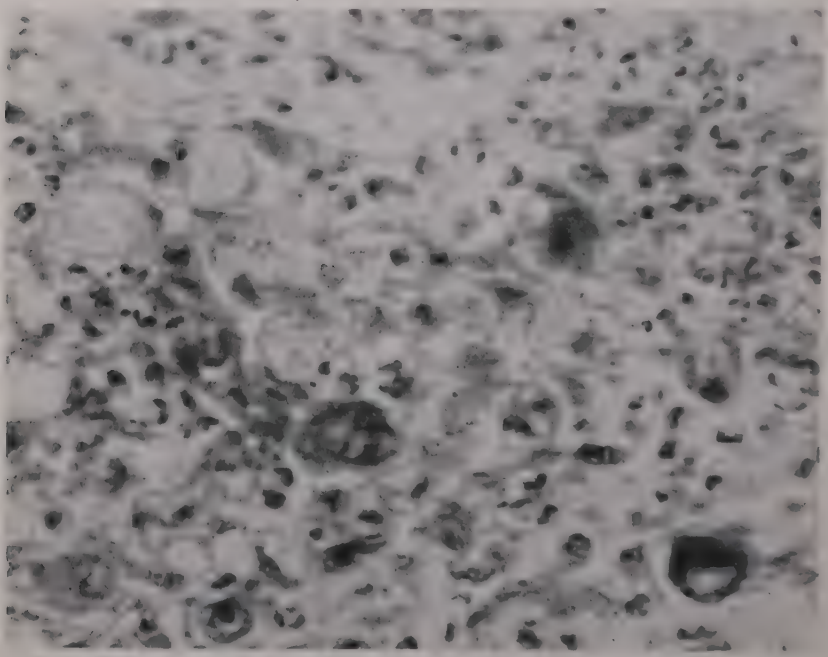


FIGURE 5. Mouse sarcoma 180 on the chorioallantoic membrane after treatment with 4-amino PGA. Note intense vacuolation and disintegration of cells.

not appreciably enlarged. In many instances, the Sarcoma 180 tumors in the egg, while showing severe histological damage, are viable when transplanted to mice. The Ma387 shows less histological injury from the dose

of 4-amino pteroylglutamic acid than the Sarcoma 180, which is severely injured.

4-Amino pteroylglutamic acid has been reported to inhibit the growth of the Rous sarcoma in the chick.[25] We have failed, however, to prevent the growth of Rous tumor cells explanted to the chorioallantoic membrane of chick embryos by treating the embryo with a single dose of 4-amino pteroylglutamic acid, which is fatal to the embryo in 4 to 7 days.

Leukemia. The earliest reports of clinical studies on analogs of folic acid concerned the use of 4-amino-PGA (aminopterin) in patients with leukemia.[10–18] It was of interest, therefore, to study this compound and other analogs in a standardized test method.[26] With this method, it has been

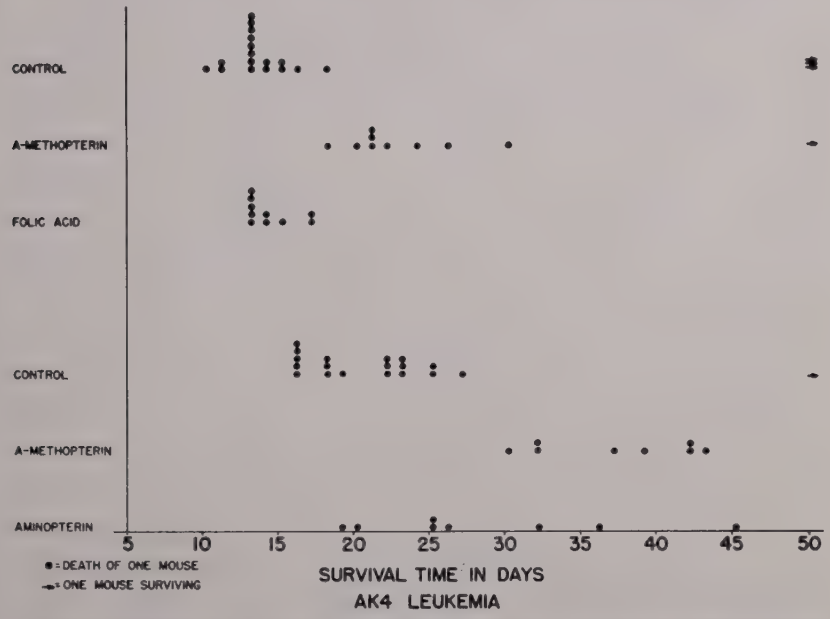


FIGURE 6. Prolongation of survival of leukemic mice treated with A-methopterin (4-amino-10-methyl folic acid) and aminopterin (4-amino pteroylglutamic acid).

possible to screen numerous substances (among them 150 compounds more or less related to folic acid) for potential use in leukemia by determining their ability to prolong the survival of the inbred strain of mice, Akm, injected with an acute lymphoid leukemia, Ak4. Other strains of leukemia have been used in supplementary tests. The enlarged liver, spleen, and lymph nodes are prominent aspects of the disease in the mouse when it is acutely ill with transmitted leukemia.

In the screening test, Akm mice are injected intraperitoneally with 0.1 cc. of a saline suspension containing 1,000,000 cells of leukemic spleen. Forty-eight hours later, the mice are divided into groups of 10 each. One group remains untreated for control on the time of death, and one group is treated with a known active compound to determine the susceptibility of

the injected cells to an effective agent. The remaining mice are treated in groups of 10 for each compound, which is injected intraperitoneally in maximum tolerated doses three times weekly for 10 doses. The mice are observed for the development of leukemia and autopsied at death for evidence of leukemia.

The results obtained with 4-amino pteroylglutamic acid, 4-amino-N^{10}-methyl pteroylglutamic acid, 4-amino pteroylaspartic acid, and 2,6-diaminopurine are presented in FIGURES 6 and 7. FIGURE 8 shows a summary of the data obtained in studies on over 500 mice with two of the more effective compounds, 2,6-diaminopurine and 4-amino-N^{10}-methyl pteroylglutamic acid. Under these test conditions, folic acid and teropterin have shown no beneficial effect.

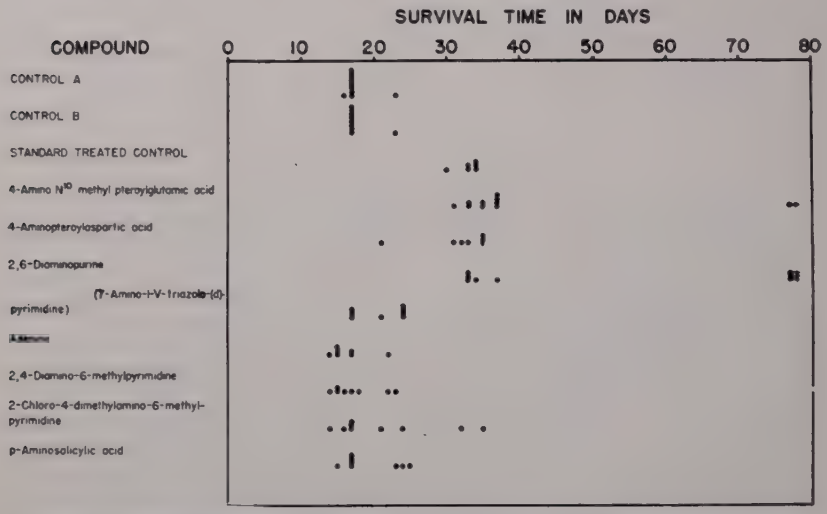


FIGURE 7. Prolongation of survival of leukemic mice treated with 4-amino-N^{10}-methyl pteroylglutamic acid, 4-amino pteroylaspartic acid, and 2,6-diaminopurine.

In an effort to obtain further information about the activity of the folic acid analogs on leukemic cells, an *in vivo* cytocidal test was utilized. This consists of administering supralethal doses of chemotherapeutic agent to donor mice to determine whether the donor cells would be affected during the remainder of their existence in the host before transplantation into susceptible recipients.

In this technique, leukemic mice were used 9–12 days after an intraperitoneal injection of a suspension of cells of leukemia Ak4. When definite splenomegaly was detectable by palpation, the mice were injected with the drugs at a series of supralethal doses. At least two donor mice were injected at each level. Mice were sacrificed 2 hours and 48 hours later and suspensions of cells from each spleen were bioassayed into four mice. TABLE 4 shows that, whereas HN2 (methyl bis (beta-chloroethyl) amine) almost completely inactivated the leukemic cells at 4 × the LD_{50} and completely

at double this dose, no effect could be demonstrated with 128, 100, and 100 times the LD_{50} of 4-amino pteroylglutamic acid, 4-amino-N^{10}-methyl pteroylglutamic acid, and 2,6-diaminopurine, respectively. This would appear to indicate a difference in mechanism of the effect of the antifolics and that of the nitrogen mustards in mouse leukemia.

Solid Tumor Screening. Another method of testing for effects against abnormal tissue in the host has been the study of inhibition of development of Sarcoma 180 in mice. This test has been used routinely in our laboratories for screening over 800 compounds for antitumor activity.[27] The procedure is as follows. Mice (CFW or RF) weighing 18–22 gms. are used. A small fragment of tumor (approximately 1–2 mm. cube) is implanted

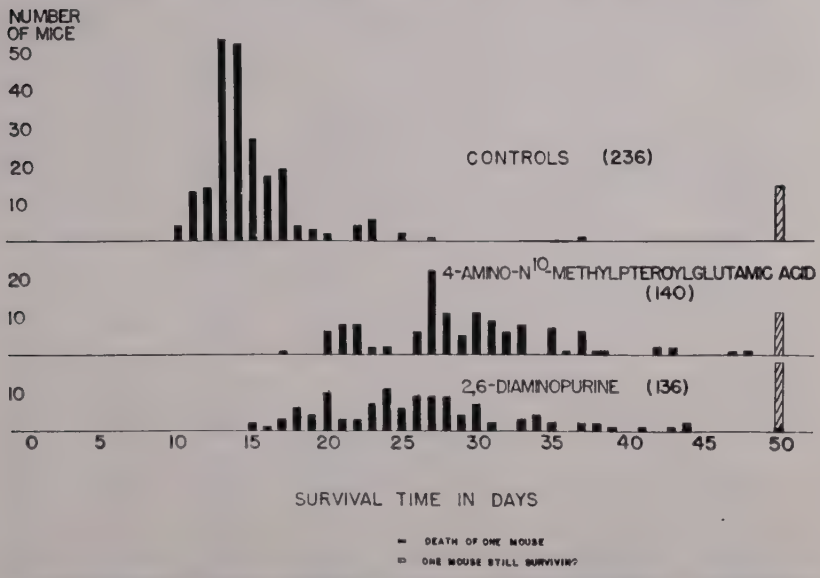


FIGURE 8. Summary of data on the treatment of leukemic mice with 4-amino-N^{10}-methyl pteroylglutamic acid and 2,6-diaminopurine.

subcutaneously by trocar into the axillary region. Twenty-four hours later, intraperitoneal injections of the test material are started in groups of five mice per substance and continued twice a day for seven days. The doses administered are the maximum tolerated on the repeated basis as determined by the Pharmacology Section.[28] At the end of the course of injections, the mice are reweighed and the tumors measured in two diameters by calipers. The amount of development of tumors in the treated animals is compared with that in the untreated controls. The tumors are considered to be markedly inhibited (+, TABLE 5) if they fail to grow or grow to approximately $\frac{1}{4}$ the average diameter of the controls. The inhibition is considered slight (±) if the tumors develop from $\frac{1}{4}$ to $\frac{3}{4}$ the diameters of the controls. Compounds in this category are unlikely to be of value therapeutically, but they are considered useful in suggesting leads for the study

of related compounds. Materials showing less activity are considered negative (−).

Compounds causing a marked inhibition of the development of Sarcoma 180 are studied further for a relation between dose and effect, for therapeutic activity, for histological evidence of damage to the tumor, and for adverse effects upon other mouse and rat tumors.

TABLE 4
In Vivo CYTOCIDAL TESTS ON LEUKEMIA AK4

Compound	LD_{50}	*Multiples of the* LD_{50}								
		1	*2*	*4*	*8*	*16*	*32*	*64*	*100*	*128*
Methyl bis-β-chloroethyl amine	5 mg./kg.	+*+ + +	++ ++	0†+ 0 +	00 00	00 00	0 0	0 0		
4-Amino pteroylglutamic acid	2 mg./kg.							+		+
4-Amino-N^{10}-methyl pteroyl-glutamic acid	50 mg./kg.	+ +	+	++ ++	++ ++	++ ++	+ 		+ +	
2,6-Diaminopurine	300 mg./kg.	+ + +		+ + +	 + +	+ + +			+ +	

* + = death of mouse on bioassay.
† 0 = survival of mouse on bioassay.

TABLE 5
INHIBITION OF GROWTH OF SARCOMA 180 BY ANALOGS OF FOLIC ACID

Compound	*Dose mg./kg./day*	*Effect**
Pteroyl triglutamic acid (Teropterin)...	2000	−
Pteroyl glutamic acid (Folic acid).....	150	−
Pteroyl aspartic acid (d).....	150	−
Pteroyl aspartic acid (dl).....	75	−
4-Amino pteroyl aspartic acid.....	30	+
4-Amino-N^{10}-methyl pteroyl glutamic acid.....	1.5	+
4-Amino pteroyl glutamic acid.....	0.2	+

* + = no development of tumor or growth to ¼ average diameter of the controls.
− = growth from ¾ diameter of control to equal growth.
± = development intermediate of those tumors classified − or +. (None in this table.)

The results in the test for inhibition of the development of Sarcoma 180 are shown in TABLE 5 for a few analogs of folic acid compared with folic acid and teropterin. We have been unable to confirm[29] the original favorable results reported elsewhere with teropterin.[30] The failure of this substance to affect Sarcoma 180 is illustrated in FIGURE 9, an area diagram showing that the tumors in treated animals developed to the same extent as in the controls. In contrast, a marked inhibition is seen with 4-amino pteroyl-glutamic acid (FIGURE 10). These results have been achieved only at levels where there was evidence of toxicity to the host, such as death, loss of

weight, and bone marrow damage, mainly a depletion of the erythroid series. This depletion has been observed with the lowest doses of the drug that show an inhibition of the tumor (FIGURE 11).

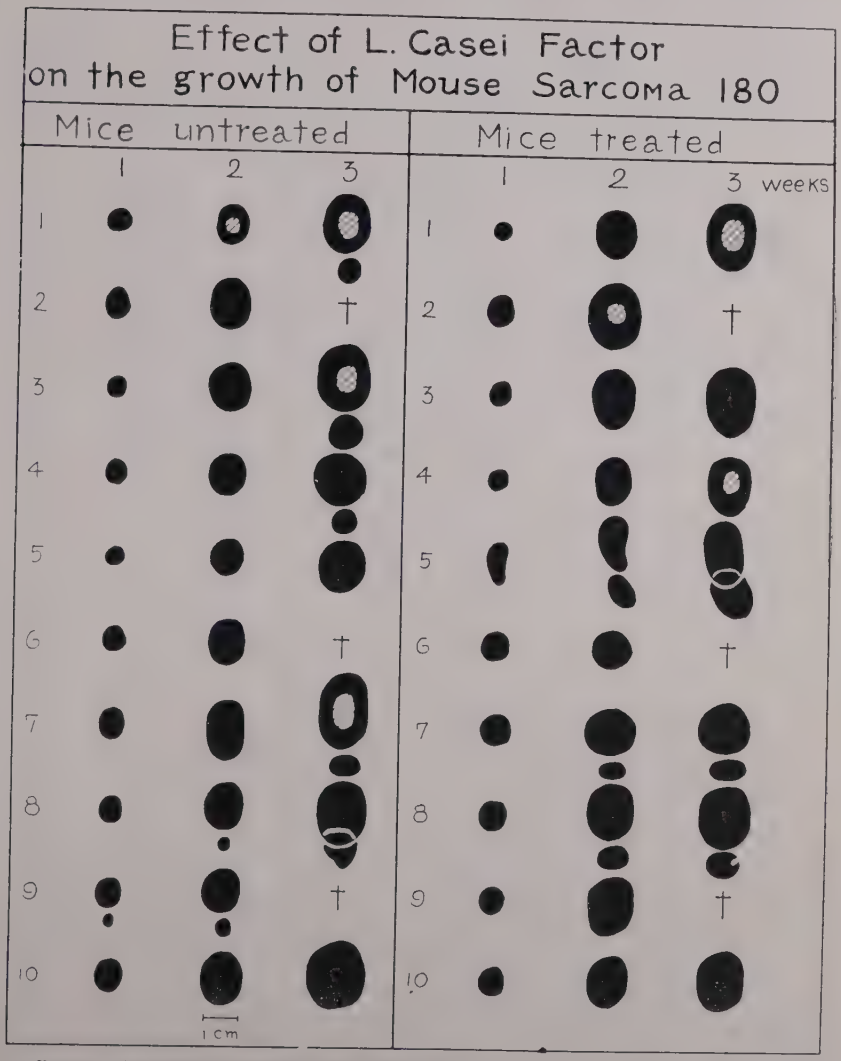


FIGURE 9. Area diagram of tumors in mice treated with *L. casei* factor (pteroyltriglutamic acid). Note lack of effect.

Damage to the tumor resulting from injections of 4-amino pteroylglutamic acid (0.25 mg./kg./day for 8 days) into the host is shown in FIGURE 12. Most of the nuclei are pycnotic and the cytoplasm is granular and often vacuolated. There is an increase in the intercellular stroma and the appearance of enlarged cells and giant nuclei.

The favorable results obtained with 4-amino-N^{10}-methyl pteroylglutamic acid were extended by studies of the inhibition obtained at various dose levels in repeated experiments. The results are summarized in TABLE 6. It is apparent that there is little or no activity at 1.0 mg./kg./day or less. Above 2.0 mg./kg./day, the toxicity is marked. At 1.5 mg./kg./day, however, marked inhibition can be observed with little evidence of toxicity, as judged by weight loss or death of the animals. The bone marrow with this compound, also, shows a depletion of the erythroid series which recovers

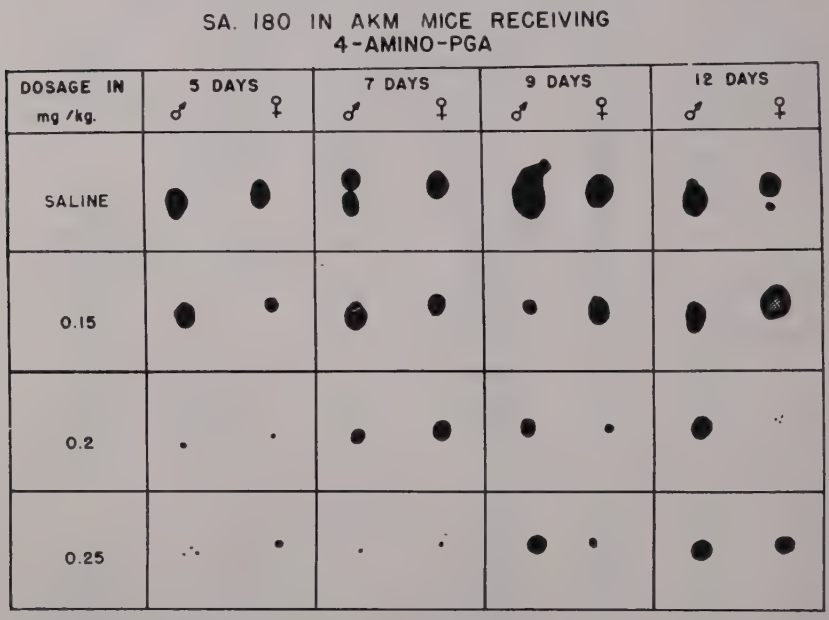


FIGURE 10. Area diagram of mice treated for 7 days with different levels of 4-amino pteroylglutamic acid. These mice are the same as those for which bone marrow studies are shown in FIGURE 11.

along with the tumor after stopping the therapy (FIGURE 11). These data illustrate the narrow range between ineffective and toxic doses. The range appears to be more favorable with 4-amino-N^{10}-methyl pteroylglutamic acid and 4-amino pteroylaspartic acid than with 4-amino pteroylglutamic acid.

When administration of the antifolics has been delayed beyond the first day after implantation of the tumor, the inhibition decreases until it becomes negligible after a delay of seven days. Maximum tolerated single doses have given slight but definite effects upon the growth of the tumor. Preliminary attempts were made to nullify the antifolic inhibition of Sarcoma 180 by simultaneous administration of folic acid. Because of the higher molar dose level it requires to achieve marked inhibition, 4-amino pteroylaspartic acid was chosen in the hope that its effect might be more readily blocked. The results are not conclusive, but as yet there has not been a striking blockage of the tumor inhibition.

The studies on 4-amino pteroylglutamic acid and 4-amino-N^{10}-methyl pteroylglutamic acid are being reported in detail.[31, 32] New leads have been

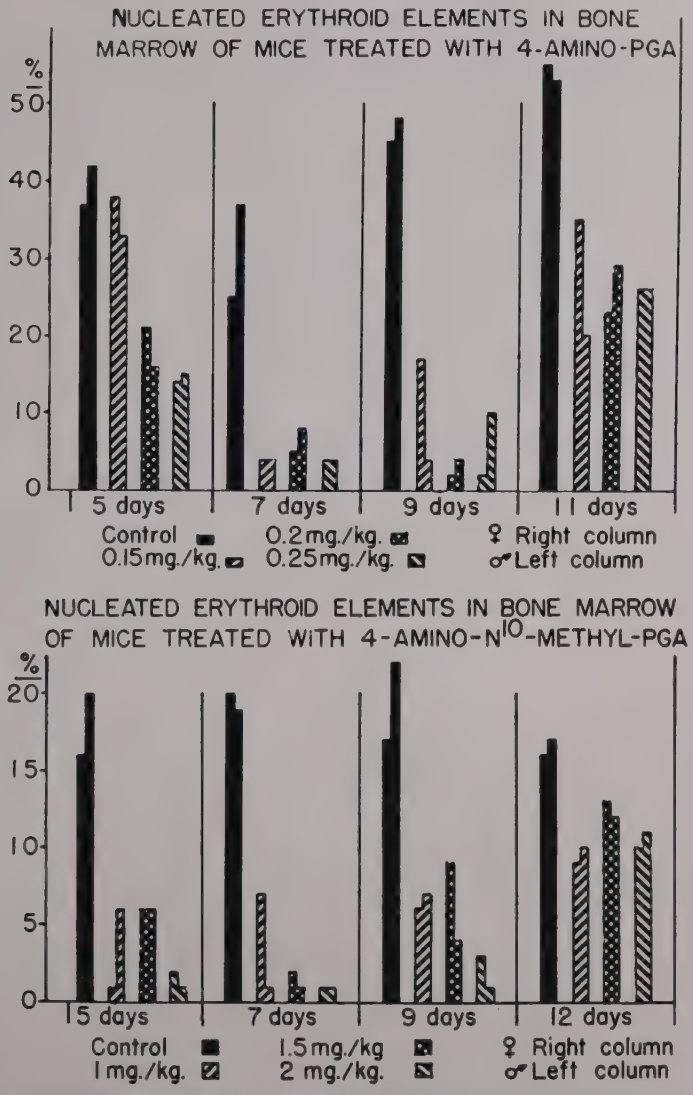
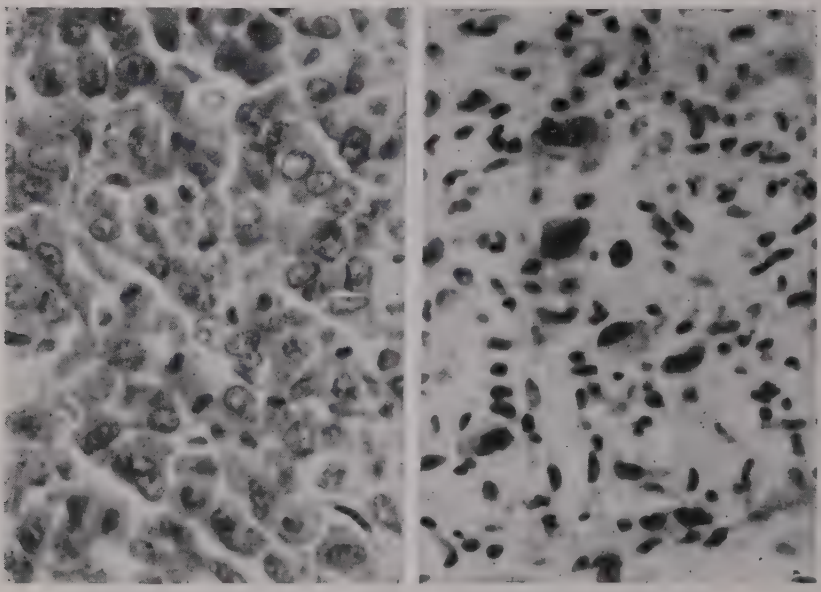


FIGURE 11. Bone marrow studies in mice bearing Sarcoma 180 treated with (a) 4-amino pteroylglutamic acid and (b) 4-amino-N^{10}-methyl pteroylglutamic acid.

sought in studies with a large number of pteridines, purines, and pyrimidines. In addition, other folic acid analogs have been studied. None of these compounds has given results as good as those with 4-amino-N^{10}-methyl pteroylglutamic acid or with 4-amino pteroylaspartic acid.

Miscellaneous Solid Tumors. The strong inhibition of Sarcoma 180 by some of the folic acid analogs made it of interest to determine the extent to which they would adversely affect spectrum of tumors including: mam-



CONTROL FOLLOWING 4-AMINO-PGA

FIGURE 12. Sarcoma 180 in mice with and without treatment with 4-amino pteroylglutamic acid. Note pycnotic nuclei and enlarged cells in the section of tumor from treated animal.

TABLE 6

INHIBITION OF GROWTH OF SARCOMA 180 BY DIFFERENT AMOUNTS OF A-METHOPTERIN

Dose mg./kg./day	*No. of mice*	*% deaths during treatment*	*Relative effect on tumor*			
			no. of groups of 5 mice showing			
			*inconclusive results**	*marked inhibition*	*slight inhibition*	*no effect*
2.5	30	47	2	4†	0	0
2.0	75	19	2	9	4	0
1.5	155	7	0	16	11	4
1.0	40	5	0	1	4	3

* Results were considered inconclusive when 3 or more mice died in any group of five.
† Means 4 groups of 5 mice each showed marked inhibition at this level.

mary adenocarcinoma EO 771; the Harding-Passey melanoma; the Wagner osteogenic sarcoma; the Patterson lymphosarcoma in mice; the Flexner-Jobling carcinoma; and sarcoma 39 in rats. The tumors were transplanted and injections of compounds once a day were made in a manner similar to that described for Sarcoma 180. The results thus far obtained with folic acid, teropterin, 4-amino PGA, 4-amino-N^{10}-methyl PGA, 4-amino PAA, and 2,6-diaminopurine are summarized in TABLE 7. The table illustrates the marked differences in susceptibility of the tumors to the compounds.

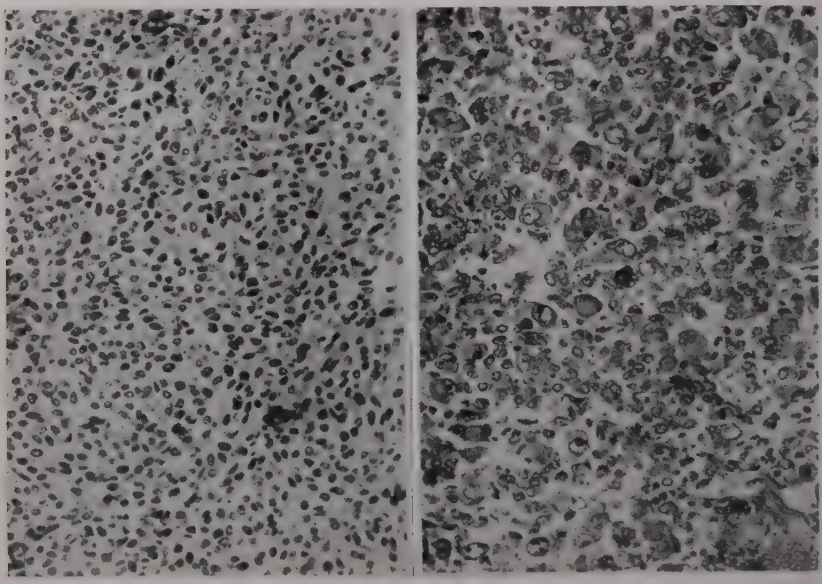
FIGURE 13. Rats given implants of rat sarcoma 39 twenty days previously. In the control rat on the left the tumor had developed normally. In the rat on the right, treated daily with 0.5 mg./kg. of 4-amino pteroylglutamic acid from the fifth through the tenth day after implantation, there is marked inhibition in the development of the tumor.

It is anticipated that such differences may be reflected in types of tumors encountered in clinical studies and that a study of these differences may be fruitful. The marked inhibition of the lymphosarcoma might have been anticipated from the information of their effectiveness in mouse leukemia.

TABLE 7

In Vivo INHIBITION OF MOUSE AND RAT TUMORS BY FOLIC ACID ANALOGS AND BY 2,6-DIAMINOPURINE

Compound	*Mouse tumors*					*Rat tumors*	
	Sarcoma 180	*Adenocarcinoma EO 771*	*H-P melanoma*	*W osteosarcoma*	*P lymphosarcoma*	*Sarcoma 39*	*F-J carcinoma*
Folic acid..... 50 mg./kg.	−	−	−	−	−	−	−
4-NH_2-PGA..... 0.25 mg./kg.	+	±	− to ±	−	+	+	−
4-NH_2-N^{10}-Me PGA..... 1.5–2.0 mg./kg.	+	±	±	−	+	+	−
4-NH_2-PAA..... 45–50 mg./kg.	+	+	−	−	+	+	−
2,6-Diaminopurine..... 60–70 mg./kg.	−	−				+	



CONTROL FOLLOWING 4 AMINO-PGA

FIGURE 14. Sections of rat sarcoma 39 from an untreated control and from a rat treated daily with 0.25 mg./kg. of 4-amino pteroylglutamic acid (aminopterin) from the sixth through the twelfth day after implantation of the tumor. In the treated tumor section, note enlargement of the tumor cells with vesicular nuclei and granular cytoplasm.

The Wagner osteogenic sarcoma and the Flexner-Jobling carcinoma are quite resistant, whereas the Rat sarcoma 39, a reticulum cell sarcoma,

is quite susceptible. It should be noted that the results on Rat sarcoma 39 with 4-amino PGA were achieved at levels quite toxic to the rat. A number died with intense diarrhea. There was a marked reduction of lymphoid tissue in the spleen, and the bone marrow showed a marked reduction of erythrocytes and in myelopoiesis. FIGURE 13 shows the marked effect of 4-amino PGA on the development of R 39. The photograph was taken 20 days after implantation of the tumor into each rat. There was essentially no development of the tumor in the rat receiving 0.25 mg./kg. on each of five consecutive days, starting five days after implantation of the tumor. FIGURE 14 shows the appearance of the untreated tumor in a section stained with hematoxylin and eosin and that of a tumor from a rat receiving 0.25 mg./kg. of 4-amino-PGA on each of six consecutive days, starting six days after implantation of the tumor. The tumor cells are enlarged with large vesicular nuclei and granular cytoplasm. There are a number of viable cells among the tumor debris. In a few instances, R 39 treated tumors have been sufficiently damaged to prevent successful transfers to other hosts.

Summary

It is evident that under a number of experimental conditions several analogs of folic acid exert an adverse effect upon tumor tissue. Some tumors are not affected by materials markedly inhibitory to other tumors. These effects are not achieved without some toxic manifestations in the host. In addition, none of the compounds at safe levels has damaged the tumor tissue sufficiently to prevent resumption of growth after administration of the drug is stopped. While the compounds leave much to be desired, they represent a step forward in the search for adequate chemotherapeutic agents for cancer.

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